Exploiting the Intergenerational Immunomodulatory Properties of *Helicobacter Pylori* for the Treatment of Allergic Disorders

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ZUSAMMENFASSUNG

Helicobacter pylori ist ein Gram-negatives, mikroaerophiles, spiralförmiges und mit Flagellen ausgerüstetes Bakterium, welches in ungefähr der Hälfte der Menschheit den Magen besiedelt. Während Tausenden von Jahren der Koexistenz mit dem Menschen hat *H. pylori* Fähigkeiten entwickelt dem angeborenen und dem adaptiven Immunsystem zu entkommen und es zu manipulieren, um dadurch in seinem Wirt zu bestehen. Obwohl dieses Bakterium als Pathogen, welches Gastritis, Magengeschwüre und letztendlich Magenkrebs verursachen kann, berüchtigt ist, konnten wir und auch andere vor Kurzem zeigen, dass es auch ein nutzbringendes Allergie-Vorbeugungspotenzial besitzt. Epidemiologische Studien haben gezeigt, dass eine Infektion mit *H. pylori* vor Asthma, anderen Allergien und vor chronisch-entzündlichen Darmerkrankungen (CED) schützen kann. Des Weiteren, konnte unser Labor in einem experimentellen Mausmodell bestätigen, dass eine Infektion die Entwicklung von allergischem Asthma und CED vorbeugt. Interessanterweise, reichte eine Behandlung mit *H. pylori*-Extrakt, VacA oder GGT (beides *H. pylori*-spezifische immunregulierende Proteine) aus um die Entwicklung von Asthma oder CED zu verhindern und war ähnlich effektiv wie eine Infektion. Die schützende Wirkung ist am stärksten wenn die Mäuse neonatal infiziert oder behandelt werden und sie ist abhängig von *H. pylori* induzierten, supprimierenden regulatorischen T Zellen (Tregs) sowie von dendritischen Zellen (DC) welche durch den Transkriptionsfaktor BATF3 kontrolliert werden und IL-10 produzieren.

Das Ziel dieser Arbeit war es, diese Erkenntnisse in einem translationalen Hausstaubmilden-induzierten Asthmamodell weiter zu entwickeln und die Auswirkung von prä- und postnatalen *H. pylori* Behandlungen des Muttertiers auf die Asthma-Prädisposition der Jungtiere zu erforschen. Zusätzlich wollte ich ermitteln ob Lebensmittelallergien - eine T_H2-dominierte Krankheit ähnlich wie allergisches Asthma - durch neonatale *H. pylori* Behandlung verhindert oder gelindert werden können. Schlussendlich war es auch unser Ziel, die den potenziellen Phänotypen zugrunde liegenden Mechanismen zu bestimmen.

In dieser Arbeit konnte ich anhand mehrerer experimenteller Mausmodelle aufzeigen, dass eine neonatale *H. pylori* Infektion, sowie Extrakt und VacA Behandlungen die Entwicklung von Lebensmittelallergien mittels eines Treg-involvierten Mechanismus lindern können, obschon die Effekte nicht so ausgeprägt und robust wie im Asthma-Modell waren. Die Behandlungen führten zu einem erhöhten Vorkommen von Tregs und einer stärkeren Demethylierung der Treg-spezifisch demethylierten Region (TSDR) und dadurch zu einer stabileren Population von Tregs. Zusätzlich konnte ich zeigen, dass prä- und postnatale, transmaternale *H. pylori* Behandlungen wirksam die Entwicklung von allergischem Asthma in den Nachkommen verhindert. Dieser Phänotyp war charakterisiert durch eine niedrigere Frequenz an DCs und $CD4^+$ T Zellen und einer erhöhten Häufigkeit von spezialisierten $ROR\gamma t^+$ und $CXCR3^+$ Treg Subpopulationen in der Lunge. Des Weiteren haben wir eine Veränderung der Mikrobiota und des epigenetischen Profils von Tregs (nämlich der TSDR Region) beobachtet. Letzteres deutet auf eine qualitative und/oder quantitative Veränderung der Stabilität und Funktionalität der Tregs hin. Die prä- und postnatale transmaternale Behandlung führte nicht zu einer generellen Immunsuppression, da eine Infektion mit Influenza Virus A die *H. pylori*-induzierte Immuntoleranz brach. Bemerkenswerterweise konnte ich zeigen, dass der transmaternal-induzierte Schutz, ohne weitere Behandlungen, auch auf die zweite Generation übertragen wird, was *H. pylori*'s Fähigkeit die Allergie-Anfälligkeit über mehrere Generation zu beeinflussen suggeriert.

SUMMARY

Helicobacter pylori is a Gram-negative, helical-shaped, flagellated and microaerophilic bacterium colonizing the human stomach of about 50 % of the world's population. During several thousand years of co-existence with humans, *H. pylori* has acquired abilities that allow it to evade and hijack both innate and adaptive branches of the immune system in order to persist in its host. Recently, we and others have shed light on the beneficial allergy-preventing potential of this bacterium, which is rather infamous for its pathogenic role in promoting gastritis, gastric ulcers and eventually gastric cancer. Epidemiological data showed the putative protective effects of *H. pylori* infection on the course of immunological diseases, including asthma, other allergic diseases, and inflammatory bowel disease (IBD). Moreover, we confirmed that in murine experimental models, *H. pylori* infection prevents the development of atopic asthma and IBD. Interestingly, tolerizing vaccination with *H. pylori*-extract, VacA or GGT, which both are immunomodulators of *H. pylori*, is as efficient as live infection in preventing asthma and IBD. The protective effects are particularly evident in mice infected or treated at an early age, and depend on *H. pylori*-mediated induction of regulatory T cells (Tregs) with highly suppressive activity as well as on IL-10 produced by specific dendritic cell (DC) subsets that are controlled by the transcription factor BATF3.

Herein, I aimed to further develop and extend these findings by using a more robust and translatable house dust mite-induced murine asthma model and by investigating the effects of maternal pre- and postnatal *H. pylori* exposure and its implication on asthma outcome in the murine offspring. Furthermore, I sought to assess whether food allergy, another typical T_H2-dominated disease, might as well be prevented by neonatal *H. pylori*-specific interventions. Finally, we strived to identify the underlying mechanism of these potential *H. pylori*-dependent immunomodulations.

In this work, I further extended the protective effects to a range of experimental food allergy models. I could show that neonatal infection, extract and VacA treatment prevents, although less efficient and robust than in the asthma model, food allergy development through a Treg-dependent mechanism. These treatments led to a higher frequency of Tregs as well as to an increased demethylation of the Treg-specific demethylated region (TSDR) and thus, more stable and committed Tregs. Additionally, I was able to show that prenatal and postnatal transmaternal *H. pylori*-treatments efficiently prevent allergic asthma development in the progeny. I characterized the associated altered immune correlates such as a decreased frequency of DCs and bulk $CD4^+$ T cells and increased frequencies of specialized $ROR\gamma t^+$ and $CXCR3^+$ Treg subsets in the lungs. Furthermore, these effects were linked to shifts in the microbiota composition, as well as the epigenetic signature of Tregs (i.e. the TSDR) indicating qualitative and/or quantitative differences in the stability and functionality of Tregs. Notably, transmaternal *H. pylori* exposure did not lead to a generalized immunosuppression due to the fact that acute infection with influenza A virus readily broke the tolerance. Most strikingly, I was able to show that the asthma-protective effects were propagated to the second generation without any further treatments, demonstrating *H. pylori*'s ability to beneficially impact allergy susceptibility of several generations.

1 Introduction

1.1 Allergic Diseases

1.1.1 Allergic asthma

1.1.1.1 Epidemiology and etiology of allergic asthma

In recent decades the prevalence of allergic asthma has dramatically increased, predominantly in developed and industrialized countries. Worldwide, around 235 million people are affected by asthma, many of them are children[1]. In Switzerland, the prevalence increased from 2% around 25 years ago to 7-15% among children and 6-7% in adults[2]. According to the WHO, 383'000 people died due to asthma in 2015, mostly in low or lower-middle income countries[1]. In the USA, annual costs caused directly and indirectly by asthma are estimated to be larger than 18 billion US$[3].
Epidemiological and experimental studies found that the increasing prevalence of asthma and also of other allergies is caused and influenced by environmental and lifestyle factors including diet, country of birth, exposure to antibiotics - especially early in life - sanitation, exposure to pets and livestock, the delivery mode, and breastfeeding, as well as genetic and epigenetic factors[4]:

Genetic factors
Numerous studies have investigated possible links between genetics and allergy, in particular asthma development. Family and twin studies as well as genome-wide association studies (GWAS) and candidate gene studies were key to identify the most important genetic variants associated with asthma.
A study investigating the association of tobacco smoke exposure and the risk of early-onset childhood asthma and adult asthma identified 12 single-nucleotide polymorphisms (SNPs) of which 7 were located on chromosome 17q21, suggesting a high importance of the region in the disease. One of these SNPs was linked to the expression of the gene *ORMDL3*, associated with transmembrane proteins in the endoplasmic reticulum[5]. However, its function in asthma development is still elusive. The linkage of this SNP with early-onset asthma was reproduced in different Asian and European cohorts[6]. Furthermore, severity of asthma and asthma exacerbations were both shown to be associated with the very same SNP underlining the relevance of the 17q21 region for asthma risk[7,8].
The interleukin-33 (IL-33)/ILRL1 pathway is known to be a major player in allergic asthma development (see chapter 1.1.1.2 Pathogenesis) and thus, multiple variants of the *IL33* and *ILRL1* genes have been elucidated to be linked to an increased risk of asthma in several GWAS and candidate gene studies[6,9]. Recent work by Traister *et al.* has revealed an association between the gene region and asthma severity[10].
The cytokine transforming growth factor-β (TGF-β) is involved in airway remodeling in the lungs and has a known role in the pathophysiology of asthma. Consequently, several *TGF-β* SNPs were found to be associated with asthma severity in children and adults[11-13].
Genetic variations within the *CH13L1* region were reported several times to be associated with disease and severity in asthma. *CH13L1*/ YKL-40 is a chitinase-like protein, which is more abundant in serum of asthma patients undergoing airway remodeling, and its levels correlate with asthma severity. Chitin is a major component of many allergens, such as house dust mites. Thus, our body produces chitinases, which degrade these foreign components. Chitinase-like proteins, which bind chitin but are unable to degrade it, are therefore associated with allergic asthma[14]. In a GWAS a

certain *CH13L1* promoter SNP was found to be linked to increased YKL-40 serum levels, asthma, bronchial hyperresponsiveness and decline in lung function in a Hutterite population of European descent and was as well predictive for asthma development during the first 5 years of life in another birth cohort study[15].
Furthermore, SNPs near the TLR-2 co-receptor genes *TLR1*, *TLR6* and *TLR10* were also shown to be associated with asthma[16].
Numerous other genetic associations with asthma have been identified, such as the *IL9*, the *IL5/IL13* region or more recently a locus at position 16q12[6,17]. Generally, the comprehensive genetic heterogeneity of asthma, the lack of studies in diverse ethnical populations, the lack of functional knowledge about newly identified genes or gene regions as well as gene-environment interactions are limiting the conclusions that can be drawn from this plethora of genetic association studies.

Environmental and lifestyle factors

The increase in prevalence of asthma and allergic diseases in general, has arrived too quickly to be accounted for by genetic alterations alone, and is more likely a combination of the latter with environmental factors, which are believed to act epigenetically.

Modern culture-independent techniques have provided a comprehensive picture of the gut microbiota, its diversity and alterations due to environmental influences[4,18]. They are based on the amplification and sequencing of parts of the *16S rRNA* gene; alternatively, metagenome or metatranscriptome sequencing is applied to obtain a higher resolution overview of the microbiota and to allow the detection of archaea, fungi, and viruses in addition to bacteria. Other emerging methods are metaproteomic and metabolomic analyses, aiming to characterize the functionality of the microbiome[19,20]. Through application of these modern culture-independent as well as culture-dependent methods, it has become increasingly clear that the composition of the gut microbiota is strongly influenced by environmental and lifestyle factors, which in turn affect the risk of allergic and other non-communicable diseases (NCDs). Better personal hygiene, smaller family size, dietary changes, and the excessive use of antibiotics in industrialized countries have all been held responsible for changes in the gut microbiota and allergy risk[21-23]. Several studies have reported beneficial effects on allergic outcomes of growing up in rural farming environments; livestock exposure, and therefore, early or prenatal microbial exposure appears to account for the lower allergy risk of farmers' children[24,25], which has been attributed mechanistically to differences in innate immune responses, as well as increased number and functions of cord blood regulatory T cells[26,27]. Moreover, bacterial communities in dust samples isolated from households with dogs or cats were found to be richer and more diverse, and exposure to such environments during infancy is known to protect against allergic disease development in childhood[4,28,29].
A reduced gastrointestinal (GI) tract microbiota diversity is clearly linked to early-onset NCDs, including atopy[30], eczema[31-34], and asthma[35]. The reduced GI microbiota diversity in allergic children is dominated by *Firmicutes*[36-38], and members of the *Bacteroidaceae* family[39] and more specifically, by increased numbers of *Bacteroides fragilis*[40,41], *Escherichia coli*, *Clostridium difficile*[34,42,43], *Bifidobacterium catenulatum*[36-38,44], and *Bifidobacterium longum*[45-47], and a lower prevalence of *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, and *Lactobacillus* species[45-48]. These general trends are not confirmed by all studies; for instance, a study in Norway found a lower rather than higher concentration of *E. coli* among allergic individuals[45]. Also, in a study by Verhulst *et al.*[49], children who developed wheezing had a lower prevalence of *C. difficile*. The reduced exposure of Western populations to microbes that are not

classically categorized as constituents of the commensal microbiota, such as the gastric colonizer and pathobiont *Helicobacter pylori*[50-52], or intestinal helminths[53,54], also increases the risk of allergic diseases[4].
Lifestyle factors such as delivery mode and breast-feeding strongly affect the establishment of the human gut microbiota and the risk of allergic outcomes. The recent finding that bacterial DNA is present in the newborn's first stool and in the fetoplacental unit suggests that the acquisition of the intestinal microbiota may already begin *in utero* and is then further shaped during birth and postnatally[55-57]. At birth, first major microbial exposures originate from the maternal vaginal and perianal microbiota. Consequently, the intestinal microbiota of newborns resembles that of his or her mother's vagina[58]. In contrast, neonates delivered by means of cesarean section (CS) acquire a gut microbiota that is similar to the one found on maternal skin. This is then followed by the typically slower (compared to vaginally delivered infants) acquisition of a more complex microbiota[59,60]. Infants born by means of CS are at higher risk for respiratory distress[61], asthma[62] and atopy, as well as obesity[63], and type I diabetes[64]. Furthermore, CS involves antibiotic exposure and can delay the onset of breastfeeding, which negatively influence the establishment of a normal healthy gut microbiota[31,65,66]. Indeed, breastfed (>4 months) neonates exhibited a reduced risk of developing asthma until 8 years of age[67]. Evidence is available that breastfeeding protects against atopic dermatitis, wheeze in early childhood and cow's milk allergy[68]. However, the data are inconsistent, as other studies have reported no benefit of breastfeeding in children from non-atopic families or in decreasing the risk of asthma in infants at 5 years of age[69,70]. Overall, the aforementioned studies imply that early-life or even prenatal exposure to microbes is critical for shaping a healthy GI tract microbiota, which can lower asthma and general allergy risk later in life[4].
More recently, the characterization of the airway microbiome has demonstrated that not only the GI tract microbes, but also the lung and airway microbiota exhibits great differences between asthmatics, non-asthmatics and those at risk for asthma[71]. A reduced microbiota diversity with an increased prevalence for Proteobacteria species was reported in patients with asthma[72]. Moreover, corticosteroid treatment-responsive patients differ in microbial lung composition from their corticosteroid-resistant counterparts[73].
Not only bacterial colonization but also viral respiratory infection early in life strongly influences the risk for allergy, in particular asthma development later in life. Infant respiratory tract infections by Respiratory syncytial virus (RSV) and human rhinovirus (HRV) are associated with a significantly higher risk for asthma development later in life as shown is various studies[74-79].

Another environmental determinant for allergy risk is the exposure to indoor allergens from mainly house dust mites (HDM), furred pets, mice, cockroaches or fungi. Already in 1990, Sporik and colleagues could demonstrate that children highly exposed to HDM during their first year of life have an elevated risk for allergic sensitization and asthma if followed to the age of 11 years[80]. Subsequent studies have further underlined the positive correlation of HDM sensitization early in life and later development of allergic rhinitis, persistent wheeze and asthma[81-84]. Lately, Baiz *et al.* and Richgels *et al.* could show that already prenatal or very early HDM exposure via the placenta or breast milk might potentiate the risk for allergic respiratory diseases[85,86]. In addition to HDM, a great body of literature could demonstrate that early-life exposure to cockroaches and mouse allergens is highly associated with allergic disease prevalence and severity, especially in urban areas[87]. In one compelling inner-city birth cohort study by Donohue *et al.*, it was even shown that mouse and cockroach sensitization increases the prevalence for atopic dermatitis, allergic rhinitis and asthma in an allergen-specific IgE dose-dependent

manner[88]. On the opposite of these aforementioned clear trends, numerous contradictory studies and systematic reviews about the association of furred pets and allergy risk exist. In summary, there is probably no increased risk associated with having pets, in one study rather a diminished risk for asthma after cat allergen exposure was reported[87]. Recently, Wegienka *et al.* focused on subgroup analyses (race, gender and delivery mode) and found different associations dependent on the subgroup. Thus, the great variability of earlier studies might be explained by the lack of subgroup division[89]. Similar to pet allergens, fungal allergens are ubiquitous in the environment and studies reporting both positive and negative correlation of fungi exposure and allergy development have been published. Visible fungal exposure such as in mold-damaged homes have been linked to an increased risk of allergic rhinitis and asthma[90]. These findings are accentuated by quantitative DNA-based[91] and β-1,3-glucan measurements, the latter showing that exposure to low levels of fungal dust is predictive for asthma in contrast to greater levels which are protective[92]. However, not the concentration alone, rather the fungal diversity might be the reason for the protective effect as recently shown by Tischer et al.[93].

Beyond the above-mentioned environmental components, air pollution such as traffic-related air pollution (TRAP) and environmental tobacco smoke (ETS) is another important factor shaping the prevalence of atopic diseases, in particular asthma. Worldwide, a plethora of studies could demonstrate how TRAP negatively affects lung development and consequently, potentiates the development of asthma and chronic obstructive pulmonary disease[94-96]. In particular, prenatal exposure (second trimester) to benzene and nitrogen dioxide was linked to an elevated risk for clinically significant low lung function, demonstrating the dramatic effects of air pollution on fetal lung development[97]. Moreover, in another study prenatal particulate matter and black carbon exposure increased the risk for wheezing by age 2 years[98], in addition to a study which found that prenatal particulate air exposure was significantly associated with asthma at age 6 years[99]. Furthermore, early-life postnatal TRAP-exposure is also impacting asthma development. A recent study by Gehring and colleagues applied meta-analyses and pooled analyses on four birth cohort studies including 14126 participants from Germany, Sweden and the Netherlands and found that exposure to pollutants early in life contributes to the development of asthma throughout childhood and adolescence[100].
However, in terms of air pollution, the strongest and most consistent pre- and postnatally-induced allergic sensitization[101] and asthma[102]-promoting effects emerge from ETS. Pooled analyses from 15 European birth cohorts demonstrated that even exposure of the mother (non-smoking) to only second hand smoke during the pregnancy will lead to an increased risk for wheezing at age 2 years, which is further increased by postnatal second hand smoke exposure and further elevated in children of atopic families[103]. Most strikingly, Li *et al.* could show that not only maternal but also grandmaternal smoking during the mother's fetal period may increase the risk for childhood asthma in the F2 offspring[104].

1.1.1.2 Pathogenesis of allergic asthma

Asthma is a chronic inflammatory disorder that affects the conducting airways leading to reversible airflow limitation, bronchial hyperresponsiveness, mucus overproduction, airway wall remodeling and/or narrowing. Patients suffer from recurrent shortness of breath, wheezing, coughing and chest tightness. Two types of asthma, allergic and non-allergic (intrinsic) asthma have been defined. The majority of asthmatic children and approximately 50% of asthmatic adults have the allergic type of asthma. Allergic

asthma is indicated by serum immunoglobulin E (IgE) antibodies and/or a positive skin prick test against inhaled allergens, most commonly HDM, but also animal dander, fungal spores, plant or tree pollen or peanuts. Intrinsic, non-allergic asthma does not involve the adaptive immune system, such as T helper 2 cells (T_H2 cells) and is not associated with allergen-specific serum IgE levels and usually develops later in life[3]. Recently, clinicians have realized that due to the diversity of the phenotypes, a more precise classification into several sub-endotypes of asthma might be superior to the above-mentioned rough division[105,106]. It is now acknowledged that the traditional definition of asthma rather embraces a collection of diseases or a syndrome and not a single disorder[107]. Recently, Wu and colleagues, found 6 different types of asthma using an unsupervised phenotyping approach[108]. Nevertheless, for the sake of brevity and relevance for this thesis, only the pathogenesis including the molecular and cellular basic immunology of the most common disease-collective called T_H2-driven or T_H2-high eosinophilic asthma will be described in the following sections.

Type 1 to 4 immune responses

Type 1, type 2 and type 3 (also called type 17) immune responses are characterized by their major cellular representatives T helper 1 cells (T_H1), T helper 2 cells (T_H2) and T helper 17 cells (T_H17), which are all subpopulations of $CD4^+$ T cells. However, also their innate counterparts, the innate lymphoid cells (ILCs) and other cell types play a major role within the three types of immunity:

Type 1 responses are initiated by IL-12 secretion of dendritic cells (DCs) and macrophages preceding the activation of natural killer cells (NK) and group 1 ILCs (ILC1s). Subsequently, T_H1 cells are activated and produce IL-2, interferon-γ (IFN-γ) and lymphotoxin-α and thus together with ILC1s and cytotoxic $CD8^+$ T cells promote type 1 immunity, characterized by intense phagocytic activity, mainly directed against intracellular threats, such as viruses, some bacteria and tumors. T_H1 and ILC1s are induced by the master regulator transcription factor T-bet, which drives the expression of other type 1-specific transcription factors and effector cytokines but also suppresses the ones from other lineages, therefore functioning as a lineage determinant[109,110].

Type 3 immunity is launched by the production of IL-1β and IL-23 by DCs and macrophages, which thereafter activates ILC3s and T_H17 cells driven by the master regulator transcription factor retinoic acid receptor-related orphan receptor-γt (ROR-γt). The subsequent effector phase is dominated by IL-22 and IL-17 secreted by lymphoid cells, antimicrobial peptides produced by epithelial cells and infiltration by neutrophils. Type 3 responses are directed at extracellular microorganisms, like most bacteria and fungi[109].

Moreover, a mixed type 1 and 3 response with lymphoid cells producing both, IFN-γ and IL-17 might occur in case of extensive tissue injury and strong inflammation[111,112].

Type 2 responses apply tissue repair mechanisms to fight against large extracellular organisms such as helminths[113]. For instance, mucus secretion and collagen deposition is induced at mucosal sites by type 2 responses. In this type of immunity epithelial cell-derived IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) production precedes the recruitment of ILC2s, eosinophils and later T_H2 cells accompanied by $IgG1^+$ and IgE^+ B cells and IL-4, IL-5 and IL-13 up-regulation[109,114]. As described for type 1 and type 3, type 2 responses are induced and maintained as well by a specific master regulator transcription factor: GATA-3 induces and drives the ILC2 and T_H2 cell lineages and simultaneously inhibits both type 1 and type 3 responses[115].

A type 4 immune response that prevents microorganisms and parasites from reaching deeper tissue sites, has also been proposed. It functions by releasing large amounts of

IgA into the intestinal lumen, tears, saliva, sweat and also the respiratory epithelium, all sites with a high bacterial or parasitic burden. IgA is the most abundantly produced antibody isotype in mammals, mainly functions through interactions with multiple receptors and blocks the microorgansims before they reach the vulnerable tissue site where they potentially would induce a type 1 or 3 response[116]. Other constituents of the type 4 immunity are antimicrobial peptides secreted by epithelial cells and the production of mucus[109].
In summary and according to Gérard Eberl's equilibrium model of immunity, all four above-mentioned arms of immunity rely on a dynamic equilibrium, well balanced in the healthy state. The four types of responses compete with each other and are in a mutually inhibitory state. Dysregulation of the equilibrium leads to pathological inflammation. Dysregulated type 1 responses are involved in systemic lupus erythematosus and type 1 diabetes. Malfunction of type 2 immunity drives allergic and pro-fibrotic pathologies, whereas type 3 responses are responsible for autoimmune inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis[109].

Regulatory T cells

Regulatory T cells (Tregs) were initially described in mice as an autoimmunity-suppressing $CD4^+$ T cell population, which expresses the IL-2 receptor α chain, CD25[117,118]. Later, Hori as well as Fontenot *et al.* found that Treg development is controlled by the transcription factor forkhead box P3 (FoxP3), which was thereafter soon established as the main marker for Tregs[119,120]. Tregs drive tolerance to self-antigens, commensal microbiota and environmental antigens, such as allergens[121,122]. Two major Treg populations exist: The thymus-derived or natural Tregs (tTregs or nTregs), which mainly control tolerance to self-antigens and the in the periphery from conventional or naive $CD4^+$ T cells derived, induced or peripheral Tregs (iTregs or pTregs), which mainly mediate tolerance to microbes and allergens. The generation of iTregs at mucosal sites requires high levels of TGF-β and retinoic acid (derived from Vitamin A), both secreted by $C103^+CD11c^+$ DCs in the intestine or by tissue resident macrophages in the lung[123,124]. Neuropilin-1 (Nrp-1) and Helios are both expressed by nTregs but not by iTregs and are therefore often used as markers to differentiate the two subsets[125,126]. nTregs and iTregs harbor distinct T cell receptor (TCR) repertoires; nTregs are rather directed against self-antigens whereas iTreg-T cell receptors are biased toward nonself antigens[127,128]. iTregs are also reported to be less stable than nTregs and can lose FoxP3 expression and consequently their lineage specific expression profile[129]. Induction and stability of FoxP3 expression and therefore of the Treg lineage is controlled by multiple signaling pathways (Figure 1) and epigenetically regulated mechanisms. TCR signaling via the nuclear factor of activated T cells (NFAT) and activator protein 1 (AP1), but also via the transcription factors cAMP-responsive-element-binding protein (CREB), activating transcription factor (ATF) and SP1 was reported to activate FoxP3 expression. Also co-stimulation, in the case of nTregs via CD28, is required for the induction of FoxP3 expression. In the periphery CD28 stimulation rather prevents iTreg differentiation. Moreover, cytokine signals triggered by receptors harboring the common cytokine-receptor γ-chain, which transmits IL-2 and other cytokine-mediated signals, are crucial for FoxP3 expression. Additionally, TGF-β-signaling via the TGF-β-receptor plays a pivotal role in Treg induction and maintenance, predominantly in iTregs in the periphery[130]. The contribution of each of these pathways may differ between Treg subsets and the exact molecular mechanism and the timing of the interplay still needs to be elucidated.

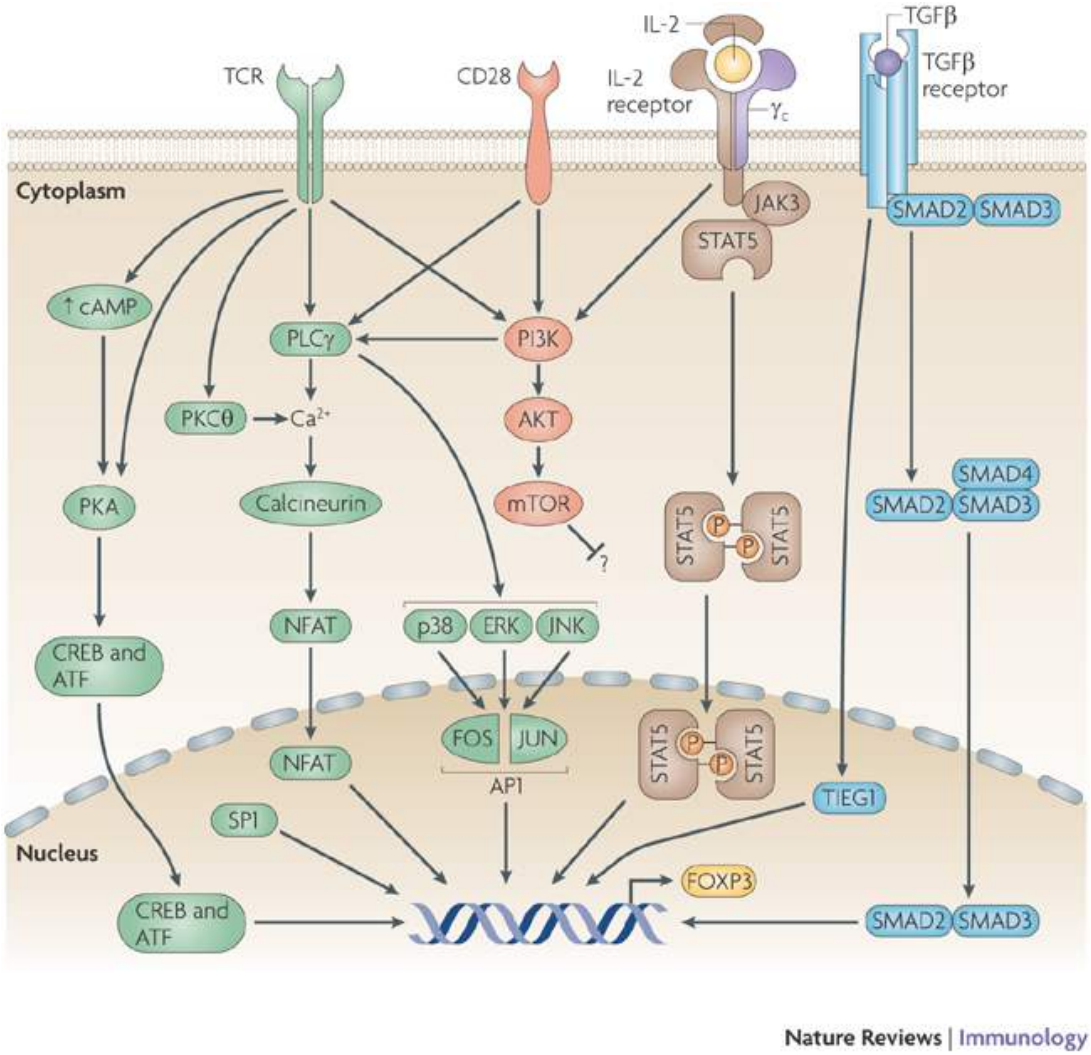

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Figure 1: Multiple signaling pathways converge to regulate FoxP3 expression. TCR firing, co-stimulation via CD28, cytokine-mediated signaling via the common cytokine-receptor γ-chain and the TGF-β-receptor all converge to induce and maintain FoxP3 expression via the trancription factors cAMP-responsive-element-binding protein (CREB), activating transcription factor (ATF), SP1, nuclear factor of activated T cells (NFAT), activator protein 1 (AP1), TGFβ - inducible early gene 1 (TIEG1), mothers against decapentaplegic homologue 3 (SMAD3) and signal transducer and activator of transcription 5 (STAT5). Adapted from [130].

Furthermore, FoxP3 expression and thus, Treg lineage stability is controlled by a region called Treg-specific demethylated region (TSDR, or also named conserved noncoding sequence 2, CNS2) containing many CpG motifs. This locus is highly demethylated in stable Tregs assuring FoxP3 expression stability and inheritance. iTregs exhibit a less hypomethylated TSDR than nTregs and are therefore less stable[129,131,132]. Other highly conserved non-coding regions that are influenced by epigenetic alterations are the *Foxp3* promoter and a TGF-β-sensor element. The *Foxp3* promoter contains CpG motifs, which are fully demethylated in Tregs compared to weakly methylated in conventional T cells. Additionally, acetylation of histones at the promoter region as well as within the TGF-β-sensor element is increased in Tregs relative to other T cells[130].
The immunosuppressive function of Tregs is mediated by secreted inhibitory cytokines (IL-10, TGF-β, IL-35) and cytolytic molecules (granzymes A and B). Additionally, deactivation of antigen presenting cells (APCs) by means of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3), deprivation of

IL-2 through CD25 as well as tuning of metabolic pathways through CD73 and CD39 are also mediators of Treg function[122].
Recently, it became apparent that Treg cells are able to further differentiate into specific effector subsets similar to conventional T cells. Evidence for T_H1-like (expressing Tbet and IFN-γ), T_H2-like (expressing GATA3 and interferon regulatory factor-4, IRF4) and T_H17-like (expressing ROR-γt and IL-17) Tregs was reported in several studies[133,134]. In summary, Tregs are a very heterogeneous cell population with a high level of plasticity. Depending on whether the cytokine environment is biased towards a type 1, type 2 or type 3 immune response, they are able to adapt and partially differentiate into these subtypes, which allows them to migrate to effector sites and dampen the associated T helper cell activity[109].

T_H2-high eosinophilic asthma

In children, T_H2-driven asthma is initiated by allergic sensitization caused by environmental factors, such as viral respiratory tract infections, exposure to oxidants like cigarette smoke or other airborne pollutants (discussed in the previous chapter) often accompanied by eczema. This activates airway epithelial cells to produce for example IL-25, IL-33 or TSLP[135]. Frequent subsequent development of allergic rhinitis can then progress to asthma in children who are susceptible due to a genetic predisposition or other less well-understood factors. This sequence of increasing symptoms is termed the atopic march[3] and leads to induction of a type 2 inflammatory cascade (Figure 2).
During sensitization, lung conventional DCs (cDCs) and epithelial cells can be directly activated by allergens. CCL2 and CCL20 are chemokines that are produced by epithelial cells in the lung upon exposure to allergens and that recruit immature pre-cDCs from the circulation[3]. IL-25, IL-33, TSLP, GM-CSF and/or IL-1α, but also danger signals like uric acid or ATP can also be released from activated epithelial cells, which favors the maturation of $CD11b^+$ cDCs, in contrast to $CD103^+$ cDCs that would induce tolerance to inhaled allergens. These $CD11b^+$ cDCs are dependent on the transcription factor IRF4 for maturation and migration to the draining lymph node[3]. Subsequent expression of co-stimulatory molecules such as the OX40 ligand further initiates migration of the $CD11b^+$ cDC to the draining lymph node (Figure 2)[135]. Upon arrival, the DC activates naive $CD4^+$ T cells via the interaction of its OX40 ligand with OX40 on the T cell and via other co-stimulatory interactions. The resulting IL-4 competent T cells migrate to B cell zones where they differentiate into T follicular helper (T_{FH}) cells prone to induce B cells to undergo IgE class-switching which leads to the production of allergen-specific IgE antibodies by differentiated plasma B cells (Figure 2)[135]. Other IL-4 competent T cells proceed into the circulation where they maturate into T_H2 cells. Subsequently, these T_H2 cells migrate to the airway epithelium and the subepithelial mucosa where they produce IL-5 and IL-13 in large quantities, inciting inflammatory and remodeling changes in the airways by recruiting and sustaining eosinophils, mast cells, basophils and goblet cells[135,136]. However, also IL-33 from epithelial cells is able to activate mast cells and basophils to secrete vasoactive amines, lipid mediators, chemokines, and cytokines like IL-4[136]. IL-33 is also able to recruit and activate ILC2 cells (Figure 2), which secrete more IL-5 and IL-13 and act in a similar way as T_H2 cells[3,135]. IL-5 is the key cytokine driving eosinophilia, recruiting eosinophils from the bone marrow, whereas IL-13 mediates the induction of chemokines, a pro-fibrogenic stromal environment, alternative macrophage activation, smooth muscle alternations enhancing airway hyper-responsiveness and goblet cell metaplasia which leads to an excess of pathological intraluminal mucus (Figure 2)[135,136]. Furthermore, IL-13 primes the vessel wall for upregulation of the adhesion molecules VCAM-1 and ICAM-1, facilitating eosinophil exit[3]. Due to a shared receptor type, IL-4 supports and contributes to the same effects as IL-13[136]. Massive infiltration of eosinophils causes airway wall

remodeling and subepithelial membrane thickening via the secretion of TGF-β[3]. The eosinophil-derived eosinophil peroxidase induces bronchial hyperreactivity and triggers adaptive immunity via effects on DCs. Moreover, activated cytolysed eosinophils are able to release extracellular DNA traps containing eosinophilic granules[3]. This might lead to high local concentrations of eosinophilic tissue-damaging agents like eosinophil-derived neurotoxin, eosinophil peroxidase and major basic protein[3]. Steadily increasing occupation of the high-affinity IgE Fc immunoglobulin receptor FcεRI on mast cells and basophils by allergen-specific IgE enhances the survival of these cells and provides a rapid mechanism for cell activation by antibody crosslinking upon further allergen exposure. Allergen-mediated FcεRI activation leads to the immediate production and release of vasoactive amines and lipid mediators that cause acute decrease of respiratory function, while also initiating the delayed phase by recruiting more inflammatory cells[136].
Overall the following pathological changes are induced: Epithelial goblet cell metaplasia, hyperplasia and excess of epithelial mucin stores, subepithelial fibrosis with increased deposition of collagen I/III and V as well as fibronectin and tenascin C, increased gland volume, changes in smooth muscle cells causing hypertrophy and hyperplasia, increased number of blood vessels. This results in the following clinical symptoms: Shortness of breath, wheeze, cough and sputum production. Exacerbations vary from mild to severe and in the worst case can result in fatal respiratory failure[135].

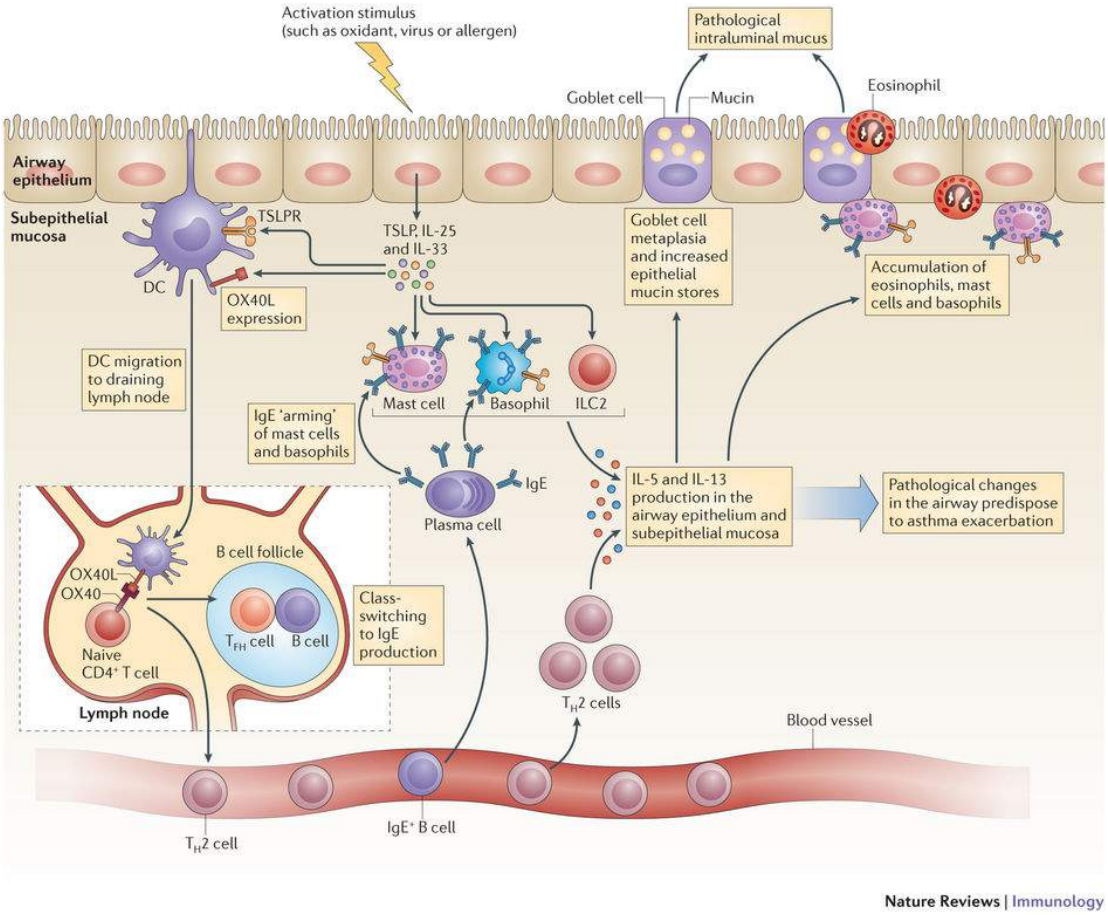

Figure 2: Type 2 pathogenesis of asthma. Epithelial-derived TSLP, IL-25 and IL-33 activates mast cells, basophils and ILC2s, in addition to DCs that migrate to the draining lymph node where they induce T_H2 and T_{FH} cell differentiation from naive $CD4^+$ T cells. T_{FH} cells induce B cell IgE class-switching in B cell zones which further leads to plasma cells producing allergen-specific IgE. T_H2 and ILC2 cells are recruited to the airway epithelium and the subepithelial mucosa and produce IL-5 and IL-13, which leads to goblet cell metaplasia and eosinophilia,

inducing pathological changes predisposing an individual to asthma and to asthma exacerbations. TSLPR, TSLP receptor; OX40L, OX40 ligand. Adapted from [135].

The persistence of type 2 immune responses initiated in childhood is quite common and its occurrence is not well understood. Most likely the involved immune cells become primed for a T_H2-dominance due to stable epigenetic changes. It is known that in the neonatal window the immune system is more susceptible for such sustainable changes that might last for an entire life or even be transferred to the next generation[137,138].

1.1.1.3 Treatment of allergic asthma

Both inhaled β2-adrenergic receptor agonists and glucocorticoids (approved in 1969 and 1974) have long been the main treatment for people suffering from asthma. Nevertheless, leukotriene receptor antagonists and IgE-directed (e.g. omalizumab, monoclonal antibody specific for IgE, by Roche/Genetech and Novartis) therapies were launched in recent years[139]. Other novel therapies, in clinical trial phase II and beyond, are IL-5, IL-13 and TSLP inhibitors. Mepolizumab is a humanized monoclonal antibody by GlaxoKlineSmith that binds and prevents IL-5 from interacting with the α-chain of the IL-5 receptor. It was shown to be effective only in a subgroup of asthma patients with persistent eosinophilia. Benralizumab by MedImmune/AstraZeneca is a humanized antibody that directly binds and blocks the α-chain of the IL-5 receptor. Reslizumab from Teva Pharmaceutical Industries is another humanized monoclonal antibody specific for IL-5. Benralizumab and reslizumab were also shown to be specifically effective in patients with persistent eosinophilia despite corticosteroid treatment. Lebrikizumab (Genetech/Roche) is an antibody that completed several phase II and III studies more or less successfully; it blocks IL-13 from binding to IL-4Rα and therefore prevents the formation of the IL-13-IL-13Rα1-IL-4Rα complex, which together with IL-13Rα2 would form the IL-13 receptor. GSK679586 from GlaxoSmithKline prevents binding of IL-13 to IL-13Rα1 and Tralokinumab from MedImmune/AstraZeneca prevents binding to IL-13Rα2, whereas Dupilumab (Regeneron Pharmaceuticals) blocks both, IL-4 and IL-13 signaling by blocking IL-4Rα. All these IL-13/IL-4 targeting medications were reported to be particulary effective in patients with evidence for type 2 inflammation[135]. Furthermore, Amgen has a TSLP inhibitor in the pipeline (AMG 157) that recently completed phase I and phase II trials. It is a fully human antibody that blocks the interaction of TSLP with its receptor and has already been shown to attenuate early- and late-phase responses to allergens[135,140].

At the moment patients suffering from very severe asthma are subjected to a combination of long-acting β2-adrenergic receptor agonists, inhaled and oral corticosteroids, leukotriene receptor antagonist, long-acting anticholinergic medication and IgE-directed therapies. Generally, the guidelines assume a positive correlation of the severity of asthma and the strength of the type 2 response because it is recommended to increase the dose of corticosteroids and add IgE-targeted medications in case of disease worsening[139]. However, such a relationship might not be true for all patients due to the existence of large subgroups that do not exhibit eosinophilia and therefore might not react to type 2-targeted therapies[141].

Another treatment method that is more and more frequently used, is allergen immunotherapy (AIT) for which steadily increasing doses of a specific allergen are applied subcutaneously or sublingually to achieve tolerance in the patient. AIT was shown to reduce symptoms, however, so far no consistent evidence for an improvement of lung function exists[142].

Overall, results from recent clinical trials and experiences from decades of glucocorticoid therapy have shown that asthma is a heterogeneous disease and that

there is a need for more personalized medical interventions including the development of robust biomarkers. Additionally, preventive measures such as reducing viral respiratory infections or reduction of exposure to antibiotics or tobacco smoke in infancy might drastically diminish asthma prevalence.

1.1.1.4 Animal models of allergic asthma

To study disease mechanisms and therapy, animal models have developed into indispensable tools. Various species have been employed to model human-like asthma, such as rats[143], guinea pigs[144], rabbits[145], sheep[146], dogs[147], primates[148,149] and mice[150]. However, the most abundantly used standard asthma models are in mice and will be discussed in the following sections.

Ovalbumin mouse models

Ovalbumin (OVA) from egg white is still the most frequently used allergen in mouse asthma models[151]. Often, BALB/c or C57BL/6 mice are used. The general OVA model involves peripheral sensitizations followed by local challenges in the airways. The sensitization usually is given via the peritoneal, subcutaneous or dermal route, consisting of adjuvanted OVA[152]. Most commonly aluminum hydroxide is used as an adjuvant to stimulate the immune system to undergo an allergen-specific type 2 polarization[153]. Other less common adjuvants are endotoxins, ozone, exogenous proteases, diesel exhaust and cigarette smoke[154]. Conrad *et al.* also described an adjuvant-free protocol[155]. Subsequent OVA challenge is carried out by intranasal or intratracheal injections or via OVA aerosols. Usually, two sensitizations distributed over two weeks, followed one week later by repetitive challenges are necessary[156]. This model, independent of the exact sensitization and challenge protocol, produces rather consistently a range of human asthma-like hallmarks in mice, such as airway hyperresponsiveness, elevated levels of IgE, airway inflammation, goblet cell hyperplasia and epithelial hypertrophy[157]. Nevertheless, the model harbors some drawbacks because it is not entirely reflecting the human situation, which usually involves a chronic inflammation of the airway wall and airway remodeling that is absent in the mouse model. Furthermore, it was shown that the hallmarks induced by some OVA models are short-lived and resolve within a few weeks after the last challenge[157]. Moreover, prolonged OVA-exposure leads to tolerance and consequently, a decreased disease phenotype[158]. Additionally, the antigen OVA is not known to be an allergen for human asthma, therefore, more recently, investigators started to use the more realistic house dust mite, grass pollen and cockroach extracts to induce asthma[159]. However, despite these concerns, the OVA mouse model allowed numerous crucial findings about disease pathogenesis and also essentially contributed to the development of therapeutic interventions[160].

House dust mite mouse models

House dust mites (HDM), belong to the most common allergens worldwide affecting 50-85% of all asthmatics in addition to the role they play in other atopic diseases like rhinitis, rhinoconjunctivitis and atopic dermatitis[160]. The HDMs, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* consist of various allergenic proteins (e.g. proteases, immunogenic epitopes, chitin) and due to their own microbiota also harbor bacterial and fungal products such as lipopolysaccharide (LPS) and β-glucan that might play a role in influencing the immune system[161-165]. The most noticed and clinically relevant HDM allergens are Der p1 and Der p2 although elucidation of the geography of HDM allergens has brought to light that the major causative allergen in a population might differ regionally and between patients[166,167].

In the HDM mouse model, distinguishing between sensitization and challenge is difficult because most models rely on the repetitive application of a standardized HDM extract without the use of an adjuvant. In the acute asthma model, on the first day, the HDM extract is usually applied intranasally or intratracheally, followed one week later by further repetitive applications of HDM extract via the same routes for approximately one week[156,160]. In contrast to the OVA model, prolonged administration of HDM extract does not lead to a tolerogenic response, instead elicited a human-like chronic type of asthma characterized by remodelling of the airways with increased mucous cell density and airway hyperreactivity, which persisted for a while after discontinuation of HDM exposure[168]. However, also the HDM model exhibits a few weaknesses, such as fewer mast cells in the epithelium of the airways in addition to lower IgE levels compared to human asthmatics in whom these features are highly elevated compared to healthy controls[150,169].

Generally, almost all mouse models induce rather short-lived type 2 inflammatory processes, which limits their translatability to approximately 50% of the patients, while the other half of asthmatics is suffering from a type 2-unrelated or mixed immune response[160]. Consequently, there is a need for a more diverse range of animal models reflecting the diversity of human asthma endotypes and their chronicity.

1.1.2 Food allergy

1.1.2.1 Epidemiology and etiology of food allergy

The exact determination of the prevalence of food allergy is challenging due to diagnostic difficulties. Often, studies rely on self-reported diagnosis, which might be affected by an increased public awareness or on serology tests, which are also known to be unreliable[170]. For instance, 25% of the US population reports that they suffer from food allergy and 40-60% of parents believe that food causes allergy in their children[171]. However, few studies applied an oral food challenge as a more reliable diagnostic measure and taken together found a prevalence of food allergy of approximately 5% in adults and 8% in children[172-174]. Similar but slightly lower numbers, 1-3% in adults and 3-8% in infants, are estimated by Brandtzaeg in an extensive review about food allergy from 2010[175]. Overall most studies reported that the prevalence of food allergy is increasing, although again, probably the data is often biased because it is derived from self-report. However, several well-designed studies have supported an increase in prevalence of food allergy over the past few decades. Using meta-regression of 20 US-based surveys representing around 400'000 children between 1988 and 2011, an increase of childhood prevalence of 1.2% points per decade was reported by Keet *et al.*[176]. Furthermore, a Chinese study used oral food challenge as a diagnostic tool in infants over 10 years and found an increase in food allergy from 3.5% to 7.7%[177]. These and other studies clearly substantiate an international increase of the prevalence of food allergy.
As described in *chapter 1.1.1.1* for allergic asthma, the increasing prevalence of food allergy is caused and influenced by environmental and lifestyle factors as well as genetic and epigenetic factors. Thus, to prevent redundancy only the most important factors differing from *chapter 1.1.1.1* are discussed in the following subchapters.

Genetic factors
As in allergic asthma, genetics and therefore atopic family history is a potent risk factor. In a large-scale study including 5'300 1-year-old infants, Koplin *et al.* could demonstrate that having one or two immediate family members with any allergic disease would increase the risk for food allergy by 40% or 80% relative to children

without an atopic family history[178]. Moreover, in 2000, Sicherer *et al.* reported that the pairwise concordance for peanut allergy was 64.3% in monozygotic twins compared to 6.8% in dizygotic twins, also suggesting a strong role for genetics[179]. Despite some studies demonstrating association of food allergy with specific gene variants, profound knowledge about specific gene involvement is scarce due to a lack of replication on a wider scale. One of the only replicated genetic susceptibility loci for food allergy is the filaggrin gene, implicating the skin as a potential route of sensitization due to its role in barrier function. Filaggrin mutations have been repetitively associated with peanut allergy[180,181] but also self-reported allergy to eggs, milk wheat and fish in a Danish population study[182]. Another rather well studied genetic implication are certain polymorphisms in the STAT6 gene, which are linked with an increase of the age of tolerance development in cow's milk allergy[183], general food sensitization[184] and risk for nut allergy[185]. Finally, two studies found that specific variants of HLA-DRB1 and HLA-DQB1 are a risk factor for food allergy. A large US GWAS found significant associations of such variants with peanut allergy but not milk or egg allergy[186]. Additionally, in a very recent study by Martino *et al.* these findings were confirmed in a cohort of Australian infants including oral food challenge as a reliable diagnostic tool for some of the participants[187].

Environmental and lifestyle factors

In the past years, the opinion that strict allergen avoidance for infants might reduce the risk for food allergy development later in life has shifted to the opposite notion, that allergen deprivation prevents early-life tolerization to oral allergens while sensitization would occur via other routes such as the skin. Most recent studies[188-190] support the latter hypothesis and putative measures are discussed below in *1.1.2.3*.

Another factor assessed in several food allergy studies, is the level of vitamin D. Indeed several studies support the hypothesis that vitamin D sufficiency or intake of vitamin D during pregnancy is associated with a decreased risk for food sensitization[191].

Factors altering or shaping the microbiome, like level of hygiene, diet, antibiotics consumptions or delivery mode might also influence the risk for food allergy development. However, a recent systematic review by Marrs *et al.* was unable to find clear evidence of a protective role for microbes in food allergy[192]. Nevertheless, many microbiome-related allergy preventive effects were observed in various studies, for details please refer to section *1.1.1.1*.

1.1.2.2 Pathogenesis of food allergy

There are non-toxic and toxic adverse reactions to foods (Figure 3). The non-toxic reaction can be divided into two main types of reactions, the non-immune-mediated (food intolerance) and the immune mediated reaction, which is per definition called food allergy. Food allergy itself is further subdivided into IgE-mediated or non-IgE-mediated food allergy. According to the Gell and Coombs classification, the IgE-mediated reaction is a type I hypersensitivity and the non-IgE-mediated reaction might be considered as a type III or IV hypersensitivity due to the involvement of IgG and IgM immune complex reactions and delayed-type or cell-mediated reactions, respectively[175]. The most common food allergens are milk, eggs, wheat, peanuts, nuts, sesame, fish but also some fruits and vegetables[193]. For the sake of brevity and relevance for this thesis, the following sections will mainly focus on IgE-mediated food allergy, for which there is a greater knowledge and understanding of the underlying immune mechanisms. Nevertheless, the problem of non-IgE-mediated food allergies should not be trivialized

due to the fact that many people suffer from such disorders (e.g. 40-50% of cow's milk allergy in children is non-IgE-mediated)[175].

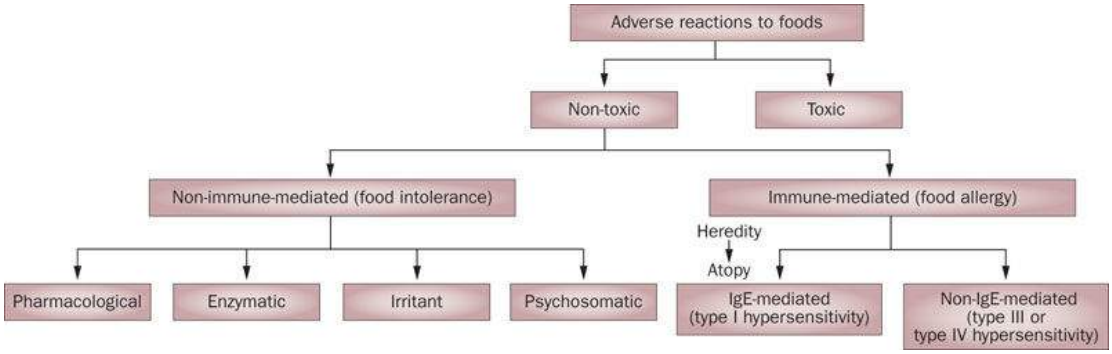

Figure 3: Classification of adverse reactions to foods. Adverse reactions to foods can be divided into toxic and non-toxic reactions, of which the latter is sub-classified into non-immune-mediated food intolerance and immune-mediated food allergy. Food allergy can be further classified into IgE-mediated type I hypersensitivity and non-IgE-mediated type III or type IV hypersensitivity. Adapted from [175].

Homeostatic anti-inflammatory defense

The mucosal immune system consists of two major anti-inflammatory mechanisms, which preserve oral tolerance to innocuous food antigens (Figure 4). On the one hand, there is immune exclusion mediated by secretory IgA (SIgA). These secretory antibodies are exported by the polymeric Ig receptor (pIgR) or also called membrane secretory component (mSC), which is cleaved apically in the epithelium, and thereby control the epithelial colonization of microorganisms[175]. More importantly, SIgA also prevents the penetration of potentially dangerous agents and thereby cooperates with innate defenses such as the mucus layer, defensins and peristalsis. Mucosal immunity is stimulated by pathogens and other antigens that are taken up through epithelial membrane cells (M cells), but also by innocuous antigens (e.g. food proteins) or commensal bacteria. On the other hand, there is hyporesponsiveness avoiding development of local and peripheral hypersensitivity against innocuous antigens and microbes. This is mediated by antigen presentation (induced by the uptake of food antigens and microbe-associated molecular patterns; MAMPs) by $CD103^+$ DCs and regulatory macrophages (see figure 4 and 5)[175]. Conversion of Vitamin A to retinoic acid by these cells leads to induction of Tregs secreting IL-10 and TGF-β and therefore suppressing a polarization towards T_H1, T_H2 or T_H17 responses (see chapter 1.1.1.2, section: Type 1 to 4 immune responses)[175]. In the skin, $CD11b^+$ DCs and Langerhans cells have also been reported to promote immune tolerance[194]. Although this adaptive oral tolerance is a very robust barrier in the adult considering the daily uptake of 130-190g food protein, it is fragile during the neonatal window and maybe longer. During that time, build-up of a well-balanced and diverse gut microbiota together with the right timing and dosing of the introduction of dietary food antigens is crucial for the establishment of the mucosal homeostatic defense[175].

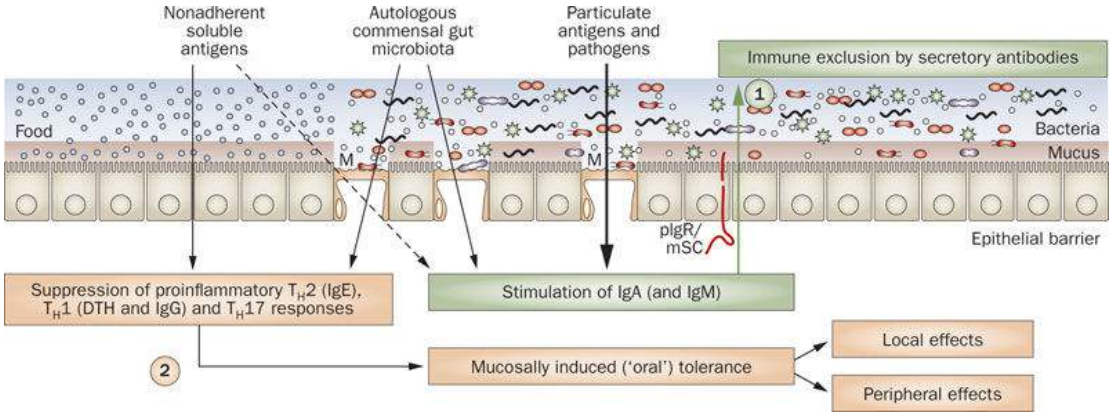

Figure 4: Anti-inflammatory mucosal defense. Two mechanisms maintain mucosal barrier function: 1) SIgA (and IgM) exported by pIgR (also called mSC) control microbiota colonization and exert immune exclusion by inhibiting penetration of harmful agents. Particulate antigens and pathogens taken up by M cells stimulate mucosal immunity (graded arrows indicate degree of stimulation). 2) However, also innocuous soluble antigens and commensal gut microbiota stimulate secretion of SIgA as well as induction of suppressive responses that inhibit polarization towards T_H1, T_H2 or T_H17. DTH, delayed-type hypersensitivity. Adapted from [175].

Sensitization to foods and development of food allergy

In the case of excessive antigenic influx and/or defective immunoregulation, hypersensitivity may be triggered and may progress to a vicious circle of proinflammatory cytokine production and epithelial cell apoptosis (Figure 5)[175]. A lot of research has focused on potential mechanisms that interfere with homeostatic immune regulation and drive this proinflammatory cycle.

In murine models it was shown that intestinal epithelial cell-derived IL-33 promotes OX40L expression on intestinal DCs, which, similar as in asthma pathogenesis, leads to induction of T_H2 cells. IL-33 has also been demonstrated to be a mediator in skin-sensitization to food antigens (major site of sensitization in peanut and milk allergy) and is also increased in damaged mouse and human skin. Upon topical allergen application it is upregulated in keratinocytes and activates the skin-draining DCs to induce a type 2 immune response[194]. ILC2s also respond to IL-33 by producing IL-4, which in turn suppresses the generation of Tregs in the small intestine. IL-33 also affects IgE-mediated activation of mast cells and therefore also enhances the acute reaction to food in addition to its sensitizing effects. Similarly, TSLP is also able to promote food allergy sensitization via OX40-L expressing DCs inducing $CD4^+$ T cells that recruit, through IL-3 secretion, basophils into the draining lymph nodes. IL-4 production by the recruited basophils promotes T_H2 skewing and supports further IL-4 production by $CD4^+$ T cells. TSLP is also able to directly influence basophil haematopoiesis. Additionally, IL-25 seems to play a similar food allergy-triggering role since it is upregulated in the intestine of mice with food allergy. Moreover, its transgenic overexpression led to enhanced experimental food allergy while lack of the IL-25 receptor resulted in resistance against food allergy. IL-25 together with T_H2 cells enhances the function of ILC2s, which amplify the immune response in experimental food allergy[194].

IL-9 has been found to be a key T_H2-cytokine for the pathogenesis of food allergy in mouse models and humans. It is a growth factor for mast cells and is much more highly expressed in memory $CD4^+$ T cells of peanut-allergic individuals compared to non-allergic controls. Moreover, a highly IL-9-IL-13-producing mucosal mast cell population, induced by T_H2 cells, was recently identified. These mast cells mediate the expansion of mature granulated mast cells in the intestine in addition to enhancing symptoms after oral challenge[194].

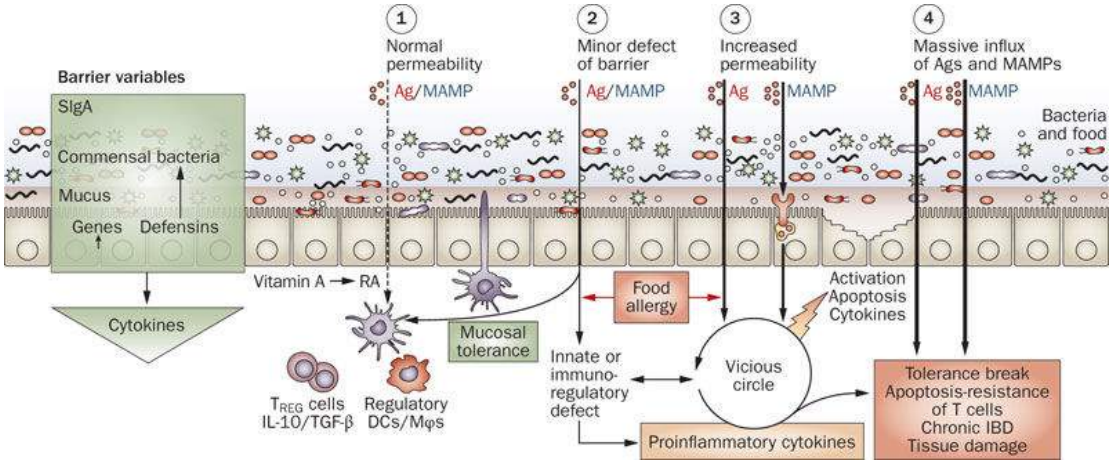

Figure 5: Stepwise abrogation of oral tolerance in the gut. 1) Normal permeability and uptake of MAMPs and antigens (Ag) by regulatory DCs and macrophages (Mφs) that convert Vitamin A to retinoic acid (RA) which leads to the induction of IL-10- and TGF-β-secreting Tregs. 2) Minor barrier defects of certain variables mentioned on the left, results in elevated influx of Ags and MAMPs. 3) If an innate or regulatory disorder exists progression into a vicious circle is possible leading to more increased permeability and apical receptor expression which results in enhanced Ag and MAMP uptake. Most probably food allergy develops between step 2 and 3 and is reversible. 4) Further aggravation elicits epithelial overactivation, apoptosis and increased secretion of proinflammatory cytokines that leads to the development of apoptosis-resistant effector T cells, chronic inflammatory bowel disease (IBD) and tissue damage. Adapted from [175].

Besides the amplification of T_H2 cell responses via IL-4, IgE-activated mast cells also suppress Treg generation and are able to reprogram their suppressive activity to a rather pathogenic IL-4 secreting cell type. Reprogrammed IL-4- and IL-13-expressing Tregs have also been described in human subjects with milk allergy, whereas in children who have outgrown their food allergy an increase of Tregs producing less IL-5 compared to allergic or healthy controls were observed[194-196].
In many food allergic individuals, the induction of the production of allergen-specific IgE is fundamental for the pathogenesis of the disease, however, the underlying mechanism and site of induction is still elusive. And despite its central role, food-specific IgE in sera does not always correlate with IgE-allergy due to occasional cross-reactivity with other allergens and the possibility of sensitization without allergy. High levels of IgE in germ-free mice were shown to be induced in the Peyer's patches in a microbiota-dependent way. However, whether IgE class-switching during sensitization to foods also occurs in the Peyers's patches remains to be determined. Probably both, memory B cells and long-lived IgE^+ plasma cells contribute to persistent antigen-specific IgE production during the course of food allergy[194].

Food-induced allergy and anaphylaxis

Food allergy provokes symptoms like atopic eczema or urticaria in the skin, the mucosa and skin can exhibit angioedema, and in the respiratory tract, laryngedema or bronchial obstruction accompanied with wheezing can occur. Symptoms are also possible in the entire digestive tract from mouth to anus (proctitis or perianal eczema), and systemic anaphylaxis can be induced. However, also unspecific symptoms like vomiting, diarrhea, nausea and constipation are typical[175].
Basophils and mast cells secrete platelet activating factor (PAF), histamine, leukotrienes, inflammatory cytokines, proteases and chemotactic molecules upon allergen challenge and thus IgE crosslinking on their surface via the high-affinity receptor FcεRI. Basophils and mast cells start degranulating within a few minutes after IgE crosslinking, leading to an immediate allergic reaction that can be fatal due to the

massive release of vasoactive mediators that might lead to vascular collapse and anaphylactic shock[197]. Consequently, basophils and mast cells are key players during allergic symptoms and anaphylaxis in mice and humans. PAF is also produced by macrophages and neutrophils that constitute parts of the so-called alternative pathways of anaphylaxis. Basophils, macrophages and neutrophils are activated through IgG-antigen immune complexes acting on FcγRIII and FcγRIV. There is also evidence for a direct allergen-induced IgE-crosslinking-dependent activation of dendritic cells and monocytes[194]. The late phase of the allergic reaction can occur several hours after allergen ingestion and consists of an influx of basophils and eosinophils. Even later (24-48h) delayed-type reactions dependent on allergen-specific T cells (activated via IgE-dependent or independent pathways) can occur[197].

1.1.2.3 Treatment of food allergy

Up to now, strict allergen avoidance and swift administration of emergency medication such as antihistamines or epinephrine in case of an accidental exposure is the only strategy to fight food allergy since no treatment has yet been approved by the food and drug administration.

However, there are some promising treatments under investigation and some of them are already far-advanced in clinical studies.

Allergen immunotherapy (AIT), already mentioned in chapter 1.1.1.3, consists of the application of steadily increasing doses of allergen with the goal to desensitize or tolerize the patient against the allergen. Several routes of allergen administration have been tested, including subcutaneous, sublingual, oral and the epicutaneous route, whereas the latter two are the most promising and clinically advanced ones. Despite the variability of the study design and a lack of very well controlled randomized, double blind, placebo-controlled studies, oral immunotherapy (OIT) seems to be effective in desensitizing about 50% of the subjects treated for egg, milk or peanut allergy[194]. It was also shown that early-life OIT might further increase the success rate. Nevertheless, adverse reactions, predominantly gastrointestinal symptoms, to OIT are frequent with 15-20% of the patients being unable to tolerate the therapy. Furthermore, patients tolerating OIT can have systemic reactions in association with acute infectious illness, exercise, or menstruation[194]. Sublingual immunotherapy (SLIT) and epicutaneous immunotherapy (EPIT), administered as drops under the tongue or as a patch directly on the skin, respectively, use lower doses and were, thus, shown to be safer. However, the increased safety comes with a decreased efficacy. Consequently, SLIT and EPIT studies usually determine the threshold dose of antigen tolerated rather than full desensitization as a measure of success and might be better suited to protect against accidental exposures and not to include the food into the diet. To generally increase the safety of AIT, recombinant allergens harboring modified epitopes, pepsin digestion or heat denaturalization are under investigation to reduce the risk for anaphylactic reactions[194].

In addition to AIT, immunomodulation has also been investigated to treat food allergy or to improve other treatment strategies. Supplementation of CpG motifs that act on Toll-like receptor 9 (TLR9) in combination with AIT was shown to be effective in mice. Chemically modified peanut allergens or DNA vaccines as immunomodulatory agents are currently in clinical trials[194].

Given the clear evidence that the microbiome influences susceptibility to food allergy probiotics are in focus of many studies. Most recent studies have been conducted using strains of *Lactobacilli* and *Bifidobacteria*, and they tend to show benefits in allergy prevention but not in the treatment of established allergies[4]. Several recent trials however, have raised new hopes that a reduction of symptoms in adult and pediatric allergic rhinitis, eczema and food allergy can be achieved by the use of probiotics[4].

Recently, a trial with peanut OIT administered together with *Lactobacillus rhamnosus* resulted in a great desensitization efficacy of 82.1% vs. 3.6% in the placebo control group. However, comparison to peanut OIT alone was not conducted. Moreover, oral fecal microbiota transplantation is currently in clinical trials[194].
IgE-inhibitors are also interesting candidates for food allergy treatment. However, most of these biologics are currently being tested for the treatment of asthma and not yet for food allergy. Omalizumab (which is already on the market for the treatment of asthma, see chapter 1.1.1.3) is currently being tested in combination with OIT to diminish side effects and treatment duration[194].
Finally, prevention rather than therapeutic treatment of existing food allergy is a crucial strategy to contain the increasing prevalence of this disease. It is now known that early oral exposure to food allergens, even in infants at risk for allergy, is preventive and exclusion of allergens rather leads to an increased sensitization, probably through the skin or airways[198,199]. Additionally, like for other allergic diseases, a reduced microbial diversity or dysbiosis are linked to an increased risk for food allergy (see chapter 1.1.2.1), and this might be prevented by reducing misuse of antibiotics or early exposure to a diverse microbial environment.

1.1.2.4 Animal models of food allergy

Most research in the field of food allergy is conducted by using experimental mouse models. In contrast to the rather well established and small variety of asthma models, there is a broad range of distinct food allergy protocols used in literature. The most important differences are mouse strain, gender, age, type of allergen, allergen formulation, sensitization route and type of adjuvant, challenge route, time schedule and dosage. A comprehensive but not complete overview can be found in the review by Liu *et al.* from 2016[200].
As mentioned earlier, genetic susceptibility is an important factor for the development of food allergy; thus, different mouse strains react differently in the same experimental protocol. BALB/c mice are known to be T_H2-biased and therefore are often used as a strain for food allergy models. However, Morafo *et al.* could demonstrate that BALB/c mice are less prone for cholera toxin (CT)-adjuvanted sensitization to peanut and milk allergens compared to C3H/HeJ and they also develop different immune responses upon challenge[201]. Therefore, BALB/c mice might be better suited for systemic sensitization and/or for mimicking allergies against other allergens. C3H/HeJ mice are frequently used to model peanut allergy because they are anaphylaxis-susceptible, probably due to a TLR4 mutation that blocks intestinal LPS signaling. However, TLR4-deficiency in BALB/c mice was not sufficient to induce strong clinical reactions to peanut, demonstrating that more differences than only the TLR4 mutation contributes to the distinct susceptibility to food allergy of BALB/c and C3H/HeJ mice. Moreover, C3H/HeOuJ mice do not respond well to peanut allergy, but are a good model organism for β-lactoglobulin-induced allergy in contrast to C3H/HeJ mice. Despite the fact that C57BL/6 mice are known to be less T_H2-biased, they are a useful strain to study mechanisms of food allergy due to the broad range of mutant strains available[200].
Mice are usually sensitized to food allergens by oral/intragastric, intraperitoneal (i.p.), or topical/epicutaneous application. However, also models using intradermal or subcutaneous injections are published. Due to the existence of oral tolerance, intragastric sensitization requires adjuvants such as the widely used CT or Staphylococcal enterotoxin B (SEB) to induce allergy. It has been suggested that CT enhances gut permeability, allergen uptake and T_H2-biased responses, however, the mechanism still remains to be elucidated[202,203]. In contrast to CT, SEB might better reflect the human pathogenesis due to its association with other allergic diseases such as atopic dermatitis and chronic rhino sinusitis[200]. SEB can also induce human food

allergy-like blood and intestinal eosinophilia in BALB/c mice, which is not observed when using CT[204]. The mechanism of action of SEB is still elusive.
Adjuvant-free intragastric sensitization has also been achieved for the first time by Chen *et. al.* in BALB/c mice[205].
The i.p.-route of sensitization has a high efficiency of systemically inducing allergen-specific IgE and IgG antibodies. Often alum is used as an adjuvant increasing locally the concentration of allergen to stimulate macrophages[206]. To screen inherent allergenic potential of novel allergens, also non-adjuvanted i.p. injections have been used[207].
Due to growing clinical evidence for the skin as a major sensitization route for food allergy, several models using epicutaneous sensitization have been established. Either adjuvant-free epicutaneous sensitization or the additional use of tape-stripping, vitamin D analogs, or intradermal injections of TSLP is applied[194].
In literature only very few food allergy mouse model protocols use intranasal sensitization although it is known that in humans sensitization might often occur via food allergen dusts through the airways[200,208].
Similar to asthma models, the sensitization phase is followed by one or several challenges with the food antigen. Most frequently are intragastric challenges, however, also many i.p.-challenged models and less frequently intravenously or intranasally challenged models can be found in the literature[200].
In conclusion, many parameters can influence the outcome of food allergy-inducing protocols in mice. Research using mouse models certainly greatly improved our understanding of the pathogenesis and treatment of food allergy. Nevertheless, the enormous variability of the distinct models used in literature, makes it difficult to compare findings and to draw an overall picture of the mechanisms involved in the course of food allergy.

1.2 Helicobacter pylori

1.2.1 Taxonomy, prevalence and transmission

The Gram-negative, helical-shaped, flagellated and microaerophilic bacterium *Helicobacter pylori* was first described, isolated and cultured by Marshall and Warren in 1982. They isolated *H. pylori* from the stomach of a patient with gastritis[209]. The human stomach is the only known natural habitat of *H. pylori* although many other *Helicobacter* species inhabiting various animals are discovered every year[210].
H. pylori co-evolved with humans over 1000s of years and has perfectly adapted to the human gastric mucosa[211]. Today, roughly half of the world's population is estimated to be infected with *H. pylori*, resulting in approximately 20% of the infected individuals in the development of peptic or duodenal ulcers, gastric adenocarcinoma or mucosa-associated lymphoid tissue lymphoma[212,213]. Consequently, the bacterium was classified as a pathogen and class I carcinogen by the WHO. For the remaining 80% of the infected individuals no clinical overt symptoms are detected[214]. In recent decades a dramatic decrease of the infection rate with *H. pylori* has been reported for industrialized and developed countries, leading to a discrepancy of the prevalence between developed (5-30%) and developing (50-90%) countries[215,216]. Hygienic conditions, family size, delivery mode, targeted and excessive use of antibiotics are some of the reasons that might explain this phenomenon[217].

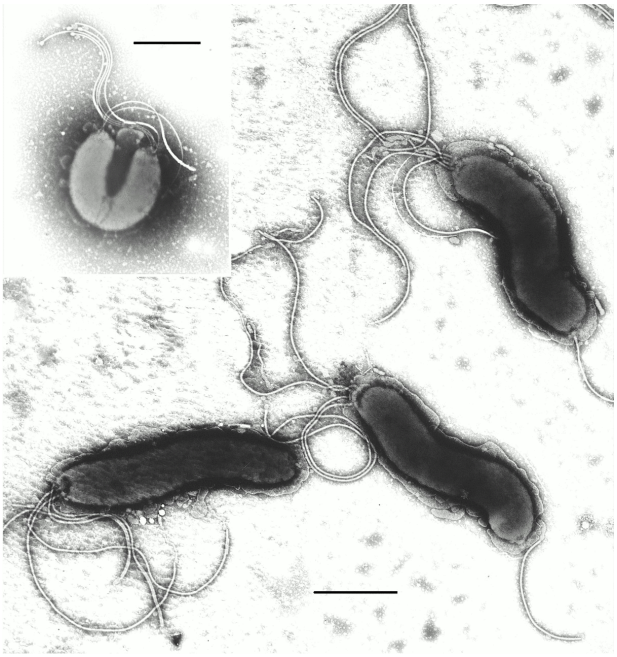

Figure 6: Electron micrographs of *Helicobacter pylori*. Non-dividing helical form of *H. pylori*, and an inset in the left upper corner showing a dividing bacterium. Scale bar 500 nm. Adapted from [218].

Most individuals are infected as infants during early childhood, after acquisition *H. pylori* can persist for a lifetime in its host[219]. However, how the transmission occurs is still elusive. Gastro-oral, oral-oral and fecal-oral are the three different routes that have been suggested to play a role.[220-222] Successful cultivation of *H. pylori* from gastric juice has been reported and thus, gastric-oral transmission might occur via this medium, most likely under poor hygienic conditions or during childhood[223,224]. Oral-oral transmission is a rather unlikely route of transfer since partners are often not infected with related strains[225,226] in addition to the questionable existence of *H. pylori* in saliva[222,227,228], although there is literature demonstrating traces of *H. pylori* in dental plaque[229]. In poor hygienic settings, fecal-oral transmission might occur due to frequent detection of *H. pylori* in human feces[230-233]. Additionally, transmission via water[234,235] or foods[236,237] is another possible route that has been proposed.

1.2.2 Virulence factors

H. pylori harbors several virulence factors mediating infection, persistence and disease development as well as severity. Vacuolating cytotoxin A (VacA), gamma-glutamyl transpeptidase (GGT), the cytotoxin-associated gene A (CagA) and outer membrane proteins are all known to be important virulence determinants, some of which will be briefly discussed in the following sections[214,238].

1.2.2.1 Vacuolating cytotoxin A

VacA is a pore-forming toxin that is able to disrupt cell polarity, to induce vacuolization and apoptosis of epithelial cells as well as to modulate the immune system by affecting T cell proliferation and effector function[239]. VacA was discovered in 1988 and is present in slightly different forms in virtually all *H. pylori* strains. It is expressed by the bacterium as a 140 kDa protoxin which is cleaved into a 88 kDa toxin upon secretion by an autotransporter mechanism, which in turn is further processed into a 33 kDa (p33) and a 55 kDa (p55) subunit[214]. VacA consists of variable regions, two of which are the signal (s) region and the mid (m) region of the gene. The s region is typed s1 or s2 and the m region is typed m1 or m2[240]. The s1m1 combination was reported to be the most

virulent and cytotoxic version in contrast to the vacuolating and non-cytotoxic s1m2 or s2m2 versions[241-245]. Other more recently discovered and not well-characterized regions are the intermediate region (i) or the deletion region (d)[246,247].

1.2.2.2 CagA and the cag pathogenicity island

The second important and one of the best-studied virulence factors is CagA, which was originally identified as a high-molecular-mass major antigen of *H. pylori* in patients infected with highly virulent strains[248,249]. CagA is 120-145 kDa and encoded on the cag pathogenicity island (cag-PAI), which is a marker for more virulent strains[250]. CagA is translocated into the eukaryotic host cell via the type IV secretion system, also encoded on the PAI, which is a needle-like macromolecular complex[251]. CagA expression, which is not evident in all strains, is linked to an increased risk for cancer development in the host, and transgenic expression of CagA in mice induced gastric carcinoma and other malignancies. Consequently, CagA is regarded as a bacterial oncoprotein[252,253]. Following translocation into the host cell, CagA is frequently phosphorylated on its tyrosine phosphorylation motifs by Src- and Abl- family kinases[253]. Phosphorylation allows the interaction of CagA with SHP-2 tyrosine phosphatase and Csk kinase while the unphosphorylated version is known to interact with Crk adaptor, c-Met, Grb2, PAR1/MARK and E-cadherin. Subsequently, these interactions result in altered cell signaling as well as changes in cell polarity, extrusion, motility, proliferation and pro-inflammatory cytokine secretion[253].

1.2.2.3 Outer membrane proteins

The outer membrane proteins HomB, BabA, IceA, SabA/B, DupA, OipA and others are mainly used for adherence of *H. pylori* to the target host cell. BabA and SabA belong to the best-characterized adhesins and exert their function by binding to Lewis antigens on the host cell surface[254,255]. These proteins are regarded as virulence factors as lack of expression renders the bacteria unable to bind to the gastric epithelial cell, thus it becomes non-pathogenic. BabA has probably evolved in response to distinct host glycosylation patterns such as the blood group of a specific region, exhibited by the fact that different strains around the world harbor distinct binding capacities to different Lewis antigens[256,257]. Generally, adherence of *H. pylori* to the gastric mucosa is important for initial and long-term colonization as well as for the strength of the inflammatory response[258].

1.2.3 Immune evasion

H. pylori is able to persist lifelong in the gastric mucosa of the infected host despite inducing a rather strong immune reaction. Various strategies, evolved over 1000s of years of adaptation to humans, are employed by the bacterium to evade and shape the host's immune response in order to achieve persistence, some of which are discussed in the following sections.

1.2.3.1 Manipulation of TLR recognition and signaling

TLRs on gastric epithelial cells and immune cells should in principle be able to detect *H. pylori* pathogen-associated molecular pattern (PAMPs) like LPS, flagellin and bacterial nucleic acids[259,260].

However, the bacterium avoids its recognition by TLRs by a number of structural modifications of LPS resulting in reduced detection and biological activity, thus endotoxicity[261-264]. Furthermore, by altering the glycosylation of the O-antigen of LPS *H. pylori* generates structures mimicking human Lewis antigens and related blood group antigens, evading the recognition by TLRs since it is being detected as self-antigen[265]. It is still elusive which TLRs are responsible for the detection of *H. pylori* LPS, some

studies propose TLR4 as a major sensor[266,267] whereas others suggest TLR2[268,269] as a main receptor.
Moreover, the bacterium further dampens recognition by TLRs by modifying residues 89-96 of the N-terminal D1 domain of its flagellin, which is essential for detection by TLR5[270,271]. In addition, *H. pylori*, possesses a less pro-inflammatory flagellar structural component, which is also not released in contrast to other bacterial flagellins[272].
Besides escaping TLR recognition, *H. pylori* is also able to manipulate TLR signaling. For instance, activation of TLR2 or TLR9 signaling by *H. pylori* components was reported to induce many anti-inflammatory effects[273].

1.2.3.2 Inhibition of CLR-mediated signaling

Another type of pattern recognition receptors, the C-type lectin receptor (CLR) is also able to interact with *H. pylori* and thus, is hijacked by it to manipulate immune responses. One of which is the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) expressed on DCs that can bind to bacterial ligands, in particular *H. pylori* Le^x and Le^y antigens which leads to suppression of T_H1 cell development[274]. Moreover, DC-SIGN ligand fucose residues of *H. pylori* can interact with the signaling complex downstream of DC-SIGN inhibiting pro-inflammatory responses[275]. Recently, it was also shown that Lewis antigens of *H. pylori* interact with the macrophage inducible C-type lectin on macrophages eliciting an anti-inflammatory immune reaction[276].

1.2.3.3 Phagocytosis and killing by reactive oxygen species and nitric oxide

H. pylori inhibits phagocytosis by monocytes and polymorphonuclear lymphocytes by using the virulence genes virB7 and virB11 and dependent on components of its type IV secretion system[277,278]. Another escape strategy by the bacterium is the α-glycosylation of cholesterol resulting in less phagocytosis by macrophages and dampened T cell activation[279,280].
Once engulfed by macrophages, *H. pylori* employs further strategies to survive phagocytosis such as delaying actin polymerization and phagosome formation and arresting phagosome maturation[281-283]. Additionally, disruption of the NADPH oxidase system, which produces reactive oxygen species (ROS) in polymorphonuclear cells, and the production of catalase and superoxide dismutase, which detoxify ROS, enables the bacterium to survive inside phagocytic cells[284-286]. In macrophages, *H. pylori* manipulates nitric oxide production, which usually kills engulfed bacteria, by employing its arginase to compete with the host cell's inducible nitric oxide synthase (iNOS) for the shared substrate L-arginine[287]. Moreover, *H. pylori* upregulates arginase II in the host cell, which results in reduced iNOS protein expression[288].

1.2.3.4 Inhibition of antimicrobial peptides

Although *H. pylori* rapidly induces gastric epithelial cells to express antimicrobial peptides such as the human beta-defensin 2 (hBD2)[289,290], hBD3[291] and the amphipathic α-helical cathelicidin LL37[292], hBD3 is downregulated in a CagA-dependent way during chronic infection[293]. Additionally, *H. pylori* exhibited resistance to hBD1 and only minimal susceptibility to hBD2 while hBD3 and LL37 efficiently killed *H. pylori*. However, the latter two antimicrobial peptides are only marginally detected in the human stomach[294].
In summary, efficient colonization of the gastric mucosa is achieved by the evolution of resistance against antimicrobial peptides as well as by manipulating the expression of those.

1.2.3.5 Interaction with and modulation of dendritic cells

H. pylori is able to manipulate the functionality of DCs, which are highly specialized antigen-presenting cells that are key to bridging innate and adaptive immune responses by presenting and transferring captured antigens, thereby inducing various T cell responses.

It was shown that phosphorylated CagA, after being taken up by DCs leads to the activation of SHP-2 which inhibits the serine/threonine protein kinase-1 (TBK-1) which in turn results in a reduced interferon regulatory factor 3 (IRF-3) translocation to the nucleus. Consequently, production of interferons is reduced, thus, leading to overall less skewing towards a T_H1 phenotype[295]. Kaebisch *et al.* have also shown that in human DCs, CagA induces a semi-mature phenotype manifesting in low expression of co-stimulatory CD86, the maturation marker CD83 and IL-12p70 and elevated expression of the tolerogenic cytokine IL-10[296].

Despite being often regarded as a pro-inflammatory cytokine, DC-mediated IL-18 secretion was reported to have tolerizing effects in *H. pylori* infection by inducing the differentiation of Tregs[297].

Furthermore, the virulence determinants VacA and GGT were shown to be essential for gastric colonization and neonatal DC-mediated tolerance *in vivo*[298]. The two factors probably exert their immunosuppressive function by inhibiting DC maturation via E2 promoter-binding factor-1[299] and in the case of GGT by glutamate-dependent inhibition of cAMP-mediated regulation of IL-6 expression[300].

1.2.3.6 Manipulation of effector T cell activity

In addition to the indirect shaping of the T cell response via the manipulation of DCs, *H. pylori* is able to directly influence T cell activity.

In the lamina propria, VacA is able to enter the human T cell cytoplasm by binding to β2 integrin; upon arrival in the cytoplasm, it interferes with the TCR-IL-2 pathway thereby inhibiting nuclear translocation of the transcription factor NFAT, thus blocking expression of T-cell-response-genes[301,302]. Moreover, the N-terminal hydrophobic region of VacA is required for the formation of membrane channels, which inhibit clonal expansion of T lymphocytes and by reducing the mitochondrial membrane potential also suppress T cell proliferation[303,304]. And, channel-independent VacA-induced actin rearrangements are able to reduce T cell activation[305].

GGT is also able to directly affect T cells by blocking T cell proliferation through G1 cell cycle arrest[306,307], depriving T cells from glutamine, reducing the expression of IL-2, CD25, IFN-γ and IL-17 as well as by altering the expression of the transcription factors c-Myc and IRF4[308].

Another mechanism of T cell manipulation is the deprivation of L-arginine required for T cell activation and function by *H. pylori's* arginase for the production of urea[309]. Additionally, cagPAI-positive strains were shown to induce the Fas ligand, thus, inducing apoptosis and limiting host immunity[310].

1.2.4 *H. pylori* and allergic diseases

Numerous epidemiological studies have addressed, and demonstrated, an inverse association of *H. pylori* infection with asthma and other allergies with respiratory tract manifestations[311-316]. This inverse association was particularly strong in children and adolescents and in individuals with early onset allergies and asthma[311-315]. The chronic inflammatory skin disease atopic dermatitis/eczema has also been inversely linked to *H. pylori* infection in studies including over 3000 German school children and almost 2000 Japanese university students[317,318]. Two meta-analyses have since been conducted that have investigated a possible inverse association of *H. pylori* with allergic asthma. Wang *et al.* retrieved 19 studies conducted up until 2012 (nine cross-sectional studies, seven

case-control studies, and three prospective cohort studies) and from these calculated a pooled OR for the association between asthma and *H. pylori* infection of 0.81[319]. A second meta-analysis -also published in 2013- which included 14 studies involving 28,283 patients, also found a significantly lower rate of *H. pylori* infection in the asthmatics than in the controls (OR=0.84, P=0.013)[316,320]. Following up on the various observational studies in human populations, mechanistic studies in experimental models have examined a possible protective effect of experimental *H. pylori* infection in animal models of allergic asthma. In a murine model of allergic asthma induced by ovalbumin or house dust mite antigen sensitization and challenge, *H. pylori* infection confers almost complete protection against the airway hyper-responsiveness, broncho-alveolar eosinophilia, lung inflammation and goblet cell metaplasia that are hallmarks of asthma in humans and mice[321]. These findings, amongst other discoveries of our lab discussed in this chapter, build the basis of my doctoral thesis. The protective effects are particularly pronounced in animals that have been experimentally infected during the neonatal period[321], i.e. at an age when humans typically contract the infection from their mothers[322]. Asthma protection conferred by *H. pylori* is abolished by antibiotic eradication therapy prior to allergen challenge, and depends critically on regulatory T-cells (Figure 7)[321]. The systemic depletion of Tregs abrogates asthma protection, and conversely, pure populations of Tregs are sufficient to transfer protection from neonatally infected donors to naive recipients. These results are in line with earlier observations that neonatal infection with *H. pylori* induces Treg-mediated immune tolerance to the bacteria[323], and that mice can be actively "tolerized" against *H. pylori* by vaccination[324]. Interestingly, the suppressive activity of Tregs in the asthma model depends on interleukin-18 proficiency of the donor[297], which is reminiscent of the prerequisites of protection against chronic intestinal inflammation. In the absence of IL-18 signaling, neonatal tolerance to the infection cannot be established; Tregs derived from IL-18$^{-/-}$ or IL-18R$^{-/-}$ donors are not protective against asthma[297]. Previous work in the lab has shown that IL-18 is produced by DCs upon exposure to *H. pylori* infection[297]. IL-18 production by DCs appears to be required for *H. pylori*-specific tolerance. IL-18 proficiency is required both in DCs derived from bone marrow and DCs isolated immunomagnetically from mesenteric lymph nodes for the conversion of naive CD4$^+$ T-cells into CD25$^+$FoxP3$^+$ Tregs[297]. In line with these observations, FoxP3$^+$ Treg numbers in the MLNs of both infected IL-18$^{-/-}$ and IL-18R$^{-/-}$ mice are significantly lower than those of infected wild type mice[297,316].

Pro-IL-18 is processed by caspase-1 to yield the mature cytokine. Several recent publications have identified the NLRP3 inflammasome as the predominant type of inflammasome to become activated upon *H. pylori* exposure of murine DCs[325-327]. TLR2 proficiency was found to be a clear prerequisite of NLRP3 inflammasome activation, as TLR2$^{-/-}$ DCs failed to activate caspase-1 and secrete caspase-1-dependent cytokines[325]. The available evidence thus points to a critical role of the TLR2/NLRP3/caspase-1/IL-18 axis in *H. pylori*-specific immune modulation, with TLR2 signaling leading to the transcriptional activation of NLRP3, which then assembles with pro-caspase-1 and the adaptor protein ASC to form the functional NLRP3 inflammasome, auto-proteolytically activate caspase-1 and process the caspase-1-dependent cytokines IL-18 and IL-1β[328]. Accordingly, TLR2-deficient mice are not protected against allergic asthma induced by house dust mite allergens[316,325].

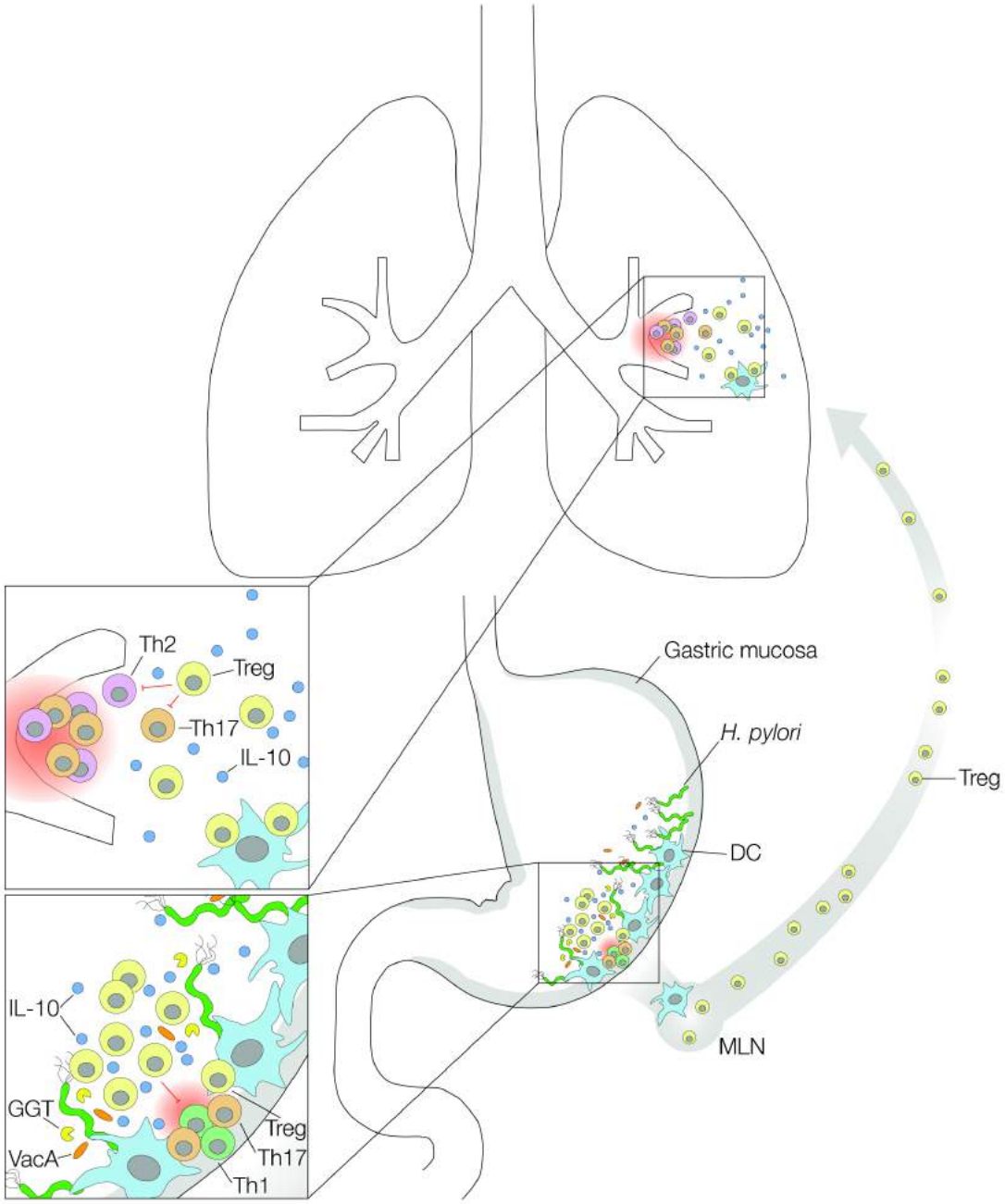

Figure 7: Gastric *H. pylori* colonization protects against allergic asthma. Despite exclusively colonizing the gastric mucosa, *H. pylori* has robust systemic effects on T cell responses in other organs. The *H. pylori* persistence factors GGT and VacA promote chronic infection by tolerizing DCs and thereby promoting local Treg differentiation (lower inset). *H. pylori*-induced Tregs and DC/Treg-derived IL-10 are required for the suppression of allergen-specific Th2 and Th17 responses in the lung (upper inset). Adapted from [316].

Several *H. pylori* determinants have been implicated in immune tolerance, the differentiation and function of suppressive Tregs, and the protection against allergic disease manifestations. In particular, the persistence factors and *H. pylori* immunomodulators vacuolating cytotoxin (VacA) and γ -glutamyl-transpeptidase (GGT) are known to be required for persistent high level colonization on the one hand, and protection against allergic asthma on the other (Figure 7)[298]. VacA- or GGT-deficient mutants fail to colonize at wild type levels, which correlates with higher numbers of T_H1 and T_H17 cells, higher expression of IFN-γ and IL-17 by restimulated mesenteric lymph

node (MLN) single cell preparations, and lower numbers of FoxP3$^+$CD25$^+$ regulatory T-cells in MLNs[298]. VacA in particular appears to bias T helper cell responses towards Tregs, which could be attributed to VacA's effects on DCs (Figure 7). DCs that were immunomagnetically purified based on their CD11c expression from the MLNs of wild-type-infected mice induced FoxP3 and CD25 expression in co-cultured naive CD4$^+$ T-cells *ex vivo*[298]. This was not observed with DCs from uninfected animals or from mice infected with a vacA mutant[298]. Interestingly, both GGT and VacA can be administered to mice in purified form and confer a level of protection against allergen-induced asthma that is comparable to the live infection (see results, Engler *et al.* 2014). Administration of several doses of VacA protects efficiently against allergen-induced asthma, especially if the protein is provided in the neonatal tolerance window. This time frame constitutes a period in both mice and humans in which immune tolerance to antigens is readily established; therefore, it is perhaps not surprising that VacA acts most potently during this time. Active tolerization against allergens using VacA requires its interaction with DCs, as mouse strains lacking IL-10 expression in the DC compartment cannot be tolerized with VacA (Figure 7)[316].

Overall, these findings impressively demonstrate *H. pylori*'s abilities to modulate the host immune system and to thereby positively affect the outcome of allergic diseases, which forms the fundament of this thesis.

2 Aims

2.1 Exploitation of the immunomodulatory properties of *H. pylori* for the development of new disease prevention and treatment strategies

Since our group could previously demonstrate that neonatal *H. pylori* infection but also treatment with VacA protects against OVA-induced allergic asthma in mice, we sought to further develop and extend these findings. Using a more robust and translatable HDM-induced murine asthma model, I evaluated the efficiency of the above-mentioned *H. pylori*-specific treatments by assessing typical hallmarks of asthma. Due to the fact that no experimental data is available about the effect of maternal pre- and postnatal *H. pylori*-specific exposure, we investigated such treatments and their implication on asthma outcome in the murine offspring.
Furthermore, we hypothesized that food allergy, another typical T_H2-dominated disease, might also be prevented by neonatal *H. pylori*-specific interventions. Hence, I established several experimental murine food allergy models and tested the effect of neonatal infection, and extract as well as VacA treatment on allergy prevention by assessing typical read-outs such as anaphylaxis score, serum levels of allergen-specific IgE, or mast cell protease 1 (MCPT-1) and T_H2 cytokine expression of re-stimulated splenocytes or mesenteric lymph node-derived cells.

2.2 Investigation of the mechanisms involved in *H. pylori*-induced immune tolerance and allergy prevention

The mechanisms involved in *H. pylori*-induced immune tolerance in the above-described experimental treatment models, in particular in food allergy and transmaternal models, are still elusive. Thus, I aimed to identify the major molecular and cellular players involved by using flow cytometric analysis and transgenic mouse models. Moreover, my goal was to identify potential epigenetic alterations induced by the aforementioned interventions. Therefore, cell sorting and subsequent gene expression analysis by qPCR as well as bisulfit-pyrosequencing to elucidate the methylation pattern of known tolerogenic loci was applied.

3 Results

3.1 Effective treatment of allergic airway inflammation with *Helicobacter pylori* immunomodulators requires BATF3-dependent dendritic cells and IL-10

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Contribution: I helped perform experiments for figures 2, 3, 4 and S4.

PNAS

Effective treatment of allergic airway inflammation with *Helicobacter pylori* immunomodulators requires BATF3-dependent dendritic cells and IL-10

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The prevalence of allergic asthma and other atopic diseases has reached epidemic proportions in large parts of the developed world. The gradual loss of the human indigenous microbiota has been held responsible for this trend. The bacterial pathogen *Helicobacter pylori* is a constituent of the normal gastric microbiota whose presence has been inversely linked to allergy and asthma in humans and experimental models. Here we show that oral or i.p. tolerization with *H. pylori* extract prevents the airway hyperresponsiveness, bronchoalveolar eosinophilia, pulmonary inflammation, and Th2 cytokine production that are hallmarks of allergen-induced asthma in mice. Asthma protection is not conferred by extracts from other enteropathogens and requires a heat-sensitive *H. pylori* component and the DC-intrinsic production of IL-10. The basic leucine zipper ATF-like 3 (BATF3)-dependent CD103$^+$CD11b$^-$ dendritic cell lineage is enriched in the lungs of protected mice and strictly required for protection. Two *H. pylori* persistence determinants, the γ-glutamyl-transpeptidase GGT and the vacuolating cytotoxin VacA, are required and sufficient for asthma protection and can be administered in purified form to prevent asthma. In conclusion, we provide preclinical evidence for the concept that the immunomodulatory properties of *H. pylori* can be exploited for tolerization strategies aiming to prevent allergen-induced asthma.

bacterial immunomodulation | allergy and asthma prevention | tolerogenic dendritic cells | bacterial persistence determinants

The prevalence of asthma and other allergic diseases has increased steadily in the course of the second half of the 20th century in both adult and pediatric, developed and developing populations (1). The lack of early childhood infections or microbial exposure due to improved sanitation, and the gradual loss of the indigenous microbiota have alternately been proposed to account for this major public health trend (2, 3). Epidemiological and experimental studies have consistently shown a strong inverse association of chronic infection with the human gastric bacterial pathogen *Helicobacter pylori* with the risk of developing allergic asthma (4–9). Chronic infection with *H. pylori* is less common in allergic individuals presenting with asthma, hay fever, or eczema than in the general population; this is especially true in children and in patients with early-onset disease (4–8). We have reported earlier that experimental infection of C57/BL6 mice with a mouse-colonizing human isolate of *H. pylori* confers robust protection against allergen-induced asthma, with particularly strong protective effects observed upon early-life exposure (9). Asthma protection could be attributed to *H. pylori*-specific tolerogenic reprogramming of dendritic cells in vitro and in vivo and to the induction of highly suppressive regulatory T cells (9, 10). Despite its striking immunomodulatory properties (11) and remarkable inverse link to various allergic diseases, the use of live *H. pylori* as a therapeutic intervention or preventive measure is unattractive due to the well-documented carcinogenic potential of chronic infection with this organism. *H. pylori* induces gastric and duodenal ulcers (12), and is also widely accepted to be the leading cause of gastric adenocarcinoma (13). Here, we have devised a strategy of *H. pylori*-specific tolerization that harnesses the bacteria's immunomodulatory properties for the prevention of asthma while avoiding the risks associated with live infection and have elucidated several key determinants of asthma protection in both the bacteria and the host.

Results

***H. pylori* Whole Cell Extract Protects Against Allergen-Induced Asthma.** To assess whether regular administration of *H. pylori* extract protects against allergen-induced asthma and thus recapitulates the effects of live infection, we treated mice with weekly doses of intragastrically administered whole cell extract from age day 7 onwards before subjecting them to ovalbumin sensitization and challenge. Control mice that had received ovalbumin but no *H. pylori* extract developed airway hyperresponsiveness to methacholine (Fig. 1 *A* and *B* and Fig. S1 *A–D*) and bronchoalveolar immune cell infiltration and eosinophilia (Fig. 1 *C* and *D*), as well as histologically evident lung inflammation and

Significance

Allergic asthma represents an increasingly common public health problem. Here, we provide preclinical evidence for the efficacy of active tolerization using *Helicobacter pylori* components as a viable strategy for asthma prevention. We use a mouse model of allergic asthma to show that regular treatment with *H. pylori* extract effectively alleviates all hallmarks of the disease. Successful treatment depends on the regulatory cytokine IL-10 and on basic leucine zipper ATF-like 3 (BATF3)-dependent dendritic cell lineages. *H. pylori* extracts lacking the γ-glutamyl-transpeptidase GGT or the vacuolating cytotoxin VacA fail to protect against asthma; conversely, both factors can be administered in purified form to achieve protection. In conclusion, the immunomodulatory properties of the common infectious agent *H. pylori* can be exploited for therapeutic purposes in an allergy model.

Author contributions: D.B.E. and A.M. designed research; D.B.E., S.R., Y.v.W., S.U., A.K., J.M., and H.M. performed research; N.Y., A.W., M.G., and T.L.C. contributed new reagents/analytic tools; D.B.E., S.R., C.T., and A.M. analyzed data; and A.M. wrote the paper.

The authors declare no conflict of interest.

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goblet cell metaplasia (Fig. 1 *E–G*). The restimulation of single cell lung preparations with ovalbumin induced the production of high levels of the Th2 cytokines IL-5 and IL-13 (Fig. 1 *H* and *I*). In contrast, mice that had received *H. pylori* extract were protected against airway hyperresponsiveness (Fig. 1 *A* and *B* and Fig. S1 *A–D*), and exhibited significantly lower levels of bronchoalveolar and pulmonary inflammation, eosinophilia and goblet cell metaplasia (Fig. 1 *C–G*). Th2 cytokine production upon allergen restimulation of lung preparations was also reduced (Fig. 1 *H* and *I*). The failure of extract-treated mice to develop allergen-induced symptoms of asthma was not due to an impaired primary response to the allergen, as the levels of ovalbumin-specific serum IgE were similar in all sensitized mice (Fig. S1*E*).

To address the specificity of the observed effects and elucidate key prerequisites of protection, we examined various administration routes and regimens, ages at treatment onset, and extracts from other gastrointestinal pathogens. Interestingly, the systemic (intraperitoneal) administration of *H. pylori* extract was as efficient as the intragastric route at conferring protection against allergen-induced asthma (Fig. S1 *F–I*). Intragastric treatment was less effective when initiated in adult mice as opposed to neonates, and four consecutive doses of extract administered to young mice before weaning were insufficient to induce full protection (Fig. S1 *F–I*). Heat-inactivated *H. pylori* extract, as well as identical amounts of extracts generated from cultures of *Escherichia coli* or *Salmonella typhimurium*, failed to confer protection against the examined hallmarks of allergic airway disease (Fig. S1 *F–I*). In conclusion, the beneficial effects of extract treatment are specific to *H. pylori* and require a heat-sensitive component of the bacteria, and are most pronounced if the treatment is initiated in young mice.

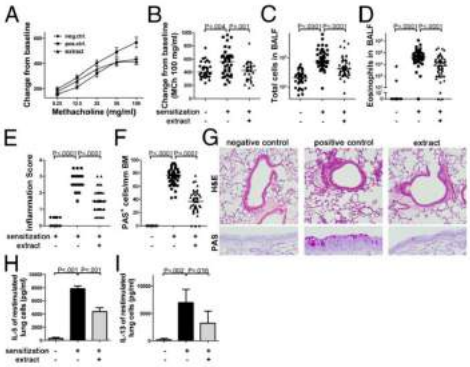

Fig. 1. Experimentally induced asthma is alleviated by treatment with *H. pylori* extract. (*A–I*) Mice were sensitized i.p. with alum-adjuvanted ovalbumin at 8 and 10 wk of age and challenged with aerosolized ovalbumin 2 wk after the second sensitization to induce asthma-like symptoms. Mock-sensitized mice served as negative controls. One group received once-weekly doses of 200 μg *H. pylori* extract intragastrically from day 7 of age until the second sensitization. (*A* and *B*) Airway hyperresponsiveness in response to increasing doses of methacholine and the highest dose of 100 mg/mL, respectively. (*C* and *D*) Total cells and eosinophils contained in 1 mL of BALF. (*E–G*) Tissue inflammation and goblet cell metaplasia as assessed on H&E and PAS-stained tissue sections; representative micrographs taken at 100× (H&E) and 400× (PAS) original magnification are shown in *G*. Pooled data from five independent studies are shown in *A–F*. (*H* and *I*) IL-5 and IL-13 secretion by single cell lung preparations restimulated with ovalbumin, as assessed by ELISA. Pooled data from two studies are shown in *H* and *I*. In scatter plots, each symbol represents one mouse; horizontal lines indicate the medians.

Successful Tolerization Against Allergen-Induced Asthma Requires IL-10 and IL-18, but Not Regulatory T Cells. *H. pylori* is known to induce the production of IL-10 in various immune cell compartments (14, 15) and high gastric levels of IL-10 ensure *H. pylori* persistence and promote *H. pylori*-specific immune tolerance (16, 17). We have shown previously that dendritic cells (DCs) play a critical role in immune tolerance to live *H. pylori*; the depletion of CD11c-positive DCs breaks tolerance and promotes clearance of the bacteria (10). To assess whether DCs produce IL-10 not only in response to live infection as shown (10), but also in response to *H. pylori* extract, cultured murine bone marrow-derived (BM) DCs were treated with increasing concentrations of extract. Indeed, BM-DCs produced and secreted large amounts of IL-10, and this was dependent on TLR2 and MyD88 signaling, but independent of TLR4 (Fig. 2*A*). A clear dose-dependent secretion of IL-10 could also be observed in human blood-derived DCs from six independent donors cultured with *H. pylori* extract (Fig. 2*B*). To address whether IL-10 is required for asthma protection conferred by extract tolerization or live infection, we administered two doses of IL-10 receptor (IL-10R)-neutralizing antibody during the challenge phase of the protocol to mice that had either received extract from the neonatal period onwards or had been infected as neonates. IL-10 signaling was required for protection against asthma in both scenarios (Fig. 2 *C–F*). We further examined the effects of extract tolerization and live infection in mice that are deficient for IL-10 production specifically in the $CD11c^+$ immune cell compartment. Although not entirely resistant to extract treatment or the beneficial effects of live infection, CD11c-Cre.$IL\text{-}10^{fl/fl}$ mice were less well protected than their Cre-negative littermates, i.e., exhibited significantly higher eosinophil counts, lung inflammation and goblet cell metaplasia (Fig. 2 *G–J*). The overall secretion of IL-10 by allergen-restimulated lung cells was reduced in CD11c-Cre.$IL\text{-}10^{fl/fl}$ mice (Fig. S2*A*), implying that $CD11c^+$ cells represent a major source of pulmonary IL-10 in this setting. In summary, we conclude that *H. pylori* extract induces IL-10 production in both murine and human DCs and that IL-10 produced by $CD11c^+$ DCs/mononuclear phagocytes, in the lungs and/or at other sites, contributes critically to protection.

Having shown previously that DC-derived IL-18 is a critical mediator of *H. pylori*-induced immune tolerance (10), we next examined the effects of *H. pylori* extract and live infection on $IL\text{-}18R^{-/-}$ mice. IL-18 signaling was absolutely required for the protective effects of live bacteria as well as extract treatment (Fig. S2 *B–E*), underscoring the tolerance-promoting role of this cytokine in the context of the *H. pylori*/host interaction. To further address whether Tregs were required for extract-mediated protection (as they are for live infection; ref. 9), we depleted $CD25^+$ Tregs (>90% depletion efficiency in the lungs, Fig. S2*F*) by applying two doses of a CD25-specific antibody before ovalbumin challenge. Treg depletion had no effect on the protection from allergic asthma conferred by *H. pylori* extract (Fig. S1 *G–J*); this result was consistent with a lack of protective activity of $CD25^+$ Tregs that were adoptively transferred from extract-treated mice to naive recipients (Fig. S1 *G–J*). *H. pylori* extract treatment of BM-DCs, in contrast to live infection, further failed to promote the expression of the Treg lineage-defining transcription factor FoxP3 in cocultured naive T cells (Fig. S2*K*), suggesting that *H. pylori* extract exerts its protective activity through the DC-intrinsic production of IL-10 (and IL-18), but independently of Tregs.

BATF3-Dependent DC Lineages Are Required for *H. pylori*-Induced Protection Against Allergic Airway Inflammation. Having identified DCs as critical mediators of *H. pylori*-specific tolerance (10) and

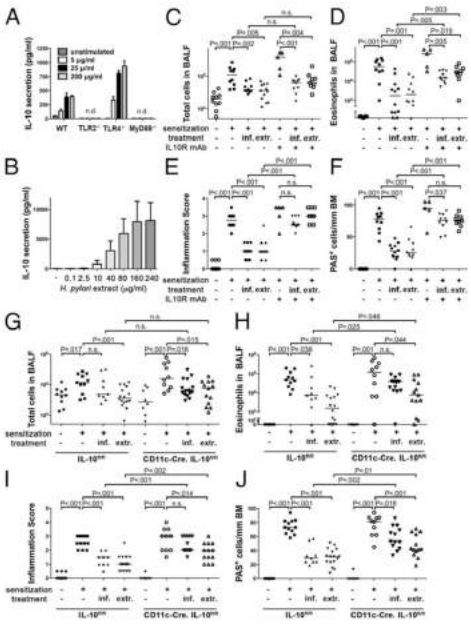

Fig. 2. IL-10 is required for *H. pylori*-induced protection against allergic asthma. (*A* and *B*) IL-10 secretion by murine bone-marrow-derived DCs of the indicated genotypes and human monocyte-derived DCs from six healthy volunteers after exposure to *H. pylori* extract. One representative experiment of three is shown in *A*, and pooled data for all six donors is shown in *B*. (*C*–*F*) Mice were treated as described in Fig. 1 or were neonatally infected with *H. pylori*; the indicated groups received 2 doses of anti-IL-10R antibody during ovalbumin challenge. (*G*–*J*) CD11c-Cre.IL-10$^{fl/fl}$ mice and their IL-10$^{fl/fl}$ littermates were either neonatally infected or received *H. pylori* extract before being subjected to ovalbumin sensitization and challenge as described in Fig. 1. Total cells (*C* and *G*) and eosinophils (*D* and *H*) contained in 1 mL of BALF. Tissue inflammation (*E* and *I*) and goblet cell metaplasia (*F* and *J*).

as key producers of protective IL-10 (Fig. 2), we next sought to dissect the role of specific DC subsets in the context of *H. pylori* infection and tolerization. To this end, we generated single cell lung preparations from extract-treated, infected, and positive as well as negative control mice and subjected them to quantitative flow cytometric analysis of various lung-infiltrating DC populations. Interestingly, despite the fact that the mice examined in this fashion exhibited very typical levels of protection (Fig. S3 *A*–*D*), their lungs were infiltrated with the same overall numbers of CD11c$^+$ MHCII$^+$ DCs as the lungs of asthmatic mice (Fig. 3*A*). However, when we distinguished between conventional and plasmacytoid DCs (cDCs, pDCs) based on their expression of B220 (also known as CD45R, a marker of the B-cell lineage that is also shared by pDCs, Fig. S3*E*), we found that CD11c$^+$ MHCII^{hi} B220$^-$ cDCs were relatively more abundant in asthmatic mice, whereas CD11c^{lo} MHCII^{lo} B220$^+$ pDCs were more abundant in the lungs of protected mice (Fig. 3 *B*–*D*). Furthermore, the total numbers of lung-infiltrating pDCs were higher than in nonsensitized negative controls (Fig. S3*F*), indicating that pDCs are actively recruited to the lungs of allergen-challenged mice that are either infected with *H. pylori* or treated with *H. pylori* extract. Another interesting difference was found among asthmatic and protected mice when we discriminated between CD11b$^+$ and CD103$^+$ cDC subsets (Fig. S3*G*). Strikingly, whereas the asthmatic lungs of positive control mice were predominantly infiltrated by CD11b$^+$ cDCs, the lungs of protected mice were relatively more infiltrated by CD103$^+$ cDCs (Fig. 3 *E*–*G*). Again, CD103$^+$ cDCs appeared to be specifically recruited to the lungs of allergen-challenged mice either infected with *H. pylori* or treated with *H. pylori* extract (Fig. S3*H*).

To assess the functional relevance of CD103$^+$ lung-infiltrating DCs in asthma protection in our model, we examined mice lacking the transcription factor basic leucine zipper ATF-like 3 (BATF3), which has previously been shown to direct the development of CD8α$^+$ lymphoid tissue DCs as well as CD103$^+$ CD11b$^-$ DCs in the lungs, intestine and skin (18). We were able to confirm that the lungs of BATF3$^{-/-}$ mice are entirely devoid of CD103$^+$ DCs, and exhibit normal and higher frequencies of pDCs and CD11b$^+$ DCs, respectively (Fig. S3 *I* and *J*). Interestingly, pure populations of mesenteric lymph node-derived DCs from BATF3$^{-/-}$ mice failed to express IL-10 upon treatment with increasing doses of *H. pylori* extract ex vivo (Fig. 3*H*). BATF3$^{-/-}$ mice were significantly less protected than wild-type mice against allergen-induced asthma upon infection with *H. pylori*, and upon treatment with *H. pylori* extract (Fig. 3 *I*–*L*), despite being colonized at comparable levels (Fig. S3*K*). In summary, BATF3-dependent CD103$^+$ DC lineages infiltrate the lungs of protected mice, and are required for the *H. pylori*-driven, IL-10-mediated protection from allergic asthma.

The *H. pylori* Persistence Determinants γ-Glutamyl Transpeptidase and Vacuolating Cytotoxin Are Required and Sufficient for Protection Against Allergic Airway Inflammation. We have shown recently that two *H. pylori* virulence determinants encoded by all clinical isolates investigated to date, the γ-glutamyl transpeptidase GGT and the vacuolating cytotoxin VacA, promote persistence through tolerogenic reprogramming of DCs (19). To examine whether GGT and/or VacA contribute to asthma protection conferred by extract tolerization, we compared the protective properties of extracts from wild-type bacteria and from GGT- or VacA-deficient isogenic mutants. Interestingly, both mutant extracts were consistently less efficient than wild-type extract at protecting allergen-sensitized and -challenged mice against bronchoalveolar and pulmonary inflammation, eosinophilia, and goblet cell metaplasia (Fig. 4 *A*–*D*). To examine whether either factor alone is sufficient to provide protection, we intraperitoneally administered either recombinant GGT or oligomeric VacA purified from culture supernatants of *H. pylori* once weekly from day 7 of age onwards. No adverse effects were observed in any of the mice, despite their young age at the time of the first doses. Strikingly, both VacA and GGT provided a level of protection against asthma that was comparable to the protection conferred by parallel whole cell extract treatment (Fig. 4 *E*–*H*). VacA was somewhat more protective than GGT at identical concentrations. VacA lacking an amino-terminal hydrophobic region of three tandem GXXXG motifs that is essential for VacA's cytotoxic activity (20) failed to protect against asthma (Fig. 4 *E*–*H*). Wild-type, but not mutant, VacA had similar effects on pulmonary Th2 cytokine production and DC infiltration (Fig. S4 *A*–*E*) as live *H. pylori* or whole cell extract (Figs. 1 and 3). IL-10R neutralization during ovalbumin challenge abrogated the protective activity of VacA, which was observed not only upon intraperitoneal administration, but also upon oral administration, and in adult as well as neonatally treated mice (Fig. S4 *F*–*I*). We conclude that GGT and VacA are key determinants of *H. pylori*-induced asthma protection and may be administered in purified form to prevent allergic asthma.

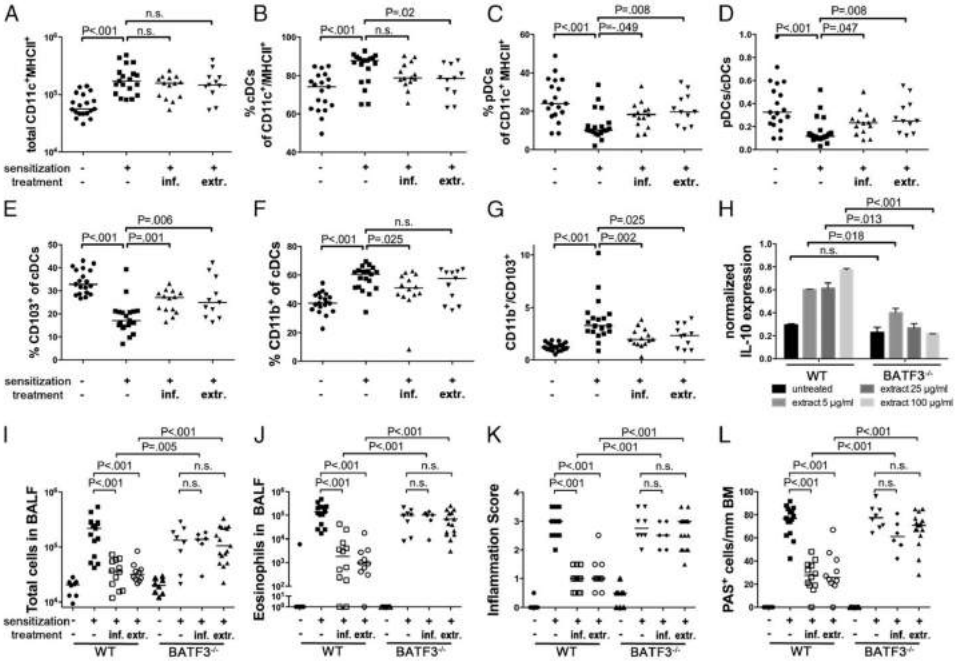

Fig. 3. CD103$^+$ conventional DCs accumulate in the lungs of *H. pylori*-infected and extract-treated mice and are required for protection. (*A–G*) Groups of mice treated as described in Figs. 1 and 2 were analyzed with respect to lung infiltration by pDCs and two lineages of cDCs. Data are pooled from three independent studies. (*A*) Total infiltration of CD11c$^+$MHC$^+$ cells. (*B* and *C*) Frequencies of B220$^-$ cDCs and B220$^+$ pDCs among all CD11c$^+$MHC$^+$ cells. (*D*) Ratios of pDCs to cDCs as calculated per mouse. (*E* and *F*) Frequencies of CD103$^+$ and CD11b$^+$ cells among all cDCs. (*G*) Ratios of CD11b$^+$ and CD103$^+$ cDCs as calculated per mouse. (*H*) IL-10 transcript levels normalized to GAPDH, of immunomagnetically isolated CD11c$^+$ DCs from mesenteric lymph nodes of WT and BATF3$^{-/-}$ mice, treated with the indicated increasing doses of *H. pylori* extract. (*I–L*) Wild-type and BATF3$^{-/-}$ mice were treated or infected as described in Figs. 1 and 2 and subjected to ovalbumin sensitization and challenge. (*I* and *J*) Total cells and eosinophils contained in 1 mL of BALF. (*K* and *L*) Tissue inflammation and goblet cell metaplasia.

Discussion

We have devised here a strategy of active tolerization for the prevention of allergic asthma that exploits the immunomodulatory properties of *H. pylori* without exposing to the risks associated with live infection. By orally or intraperitoneally administering *H. pylori* whole cell extract to allergen-sensitized mice, we were able to achieve a level of protection against asthma that was equivalent to the protection conferred by live infection (9). Extract-mediated protection was highly specific to *H. pylori*, i.e., was not conferred by extract from other Gram-negative enteropathogens such as *E. coli* or *Salmonella typhimurium*. The treatment was particularly successful when initiated in young mice, an observation that is in line with the superior protection afforded by experimental (live) infection of neonatal relative to adult mice (9). The differential susceptibility to successful tolerization of neonates and adults may be attributable to the general tolerogenic bias of the immature neonatal immune system, with its higher Treg/Teffector cell ratios and Treg-predominant responses to foreign antigens (21). Our results are in line with the epidemiological finding that children benefit more from harboring *H. pylori* than adults in terms of their asthma risk (6); similarly, early onset asthma in adolescents and young adults is more strongly inversely correlated with *H. pylori* seropositivity than adult-onset asthma (5). The data presented here thus imply that children at high risk of developing asthma are more likely than adults to benefit from *H. pylori*-specific tolerization strategies.

Having shown earlier that *H. pylori*-specific immune tolerance is a consequence of tolerogenic reprogramming of DCs by the bacteria (10), we set out to examine the contribution of specific DC lineages and their immunomodulators to immune tolerance and asthma protection in the settings of neonatal infection and neonatal-onset tolerization with *H. pylori* extract. A careful immunophenotypic analysis of the DC subsets infiltrating the lungs of protected mice revealed a preferential recruitment of CD103$^+$CD11b$^-$ conventional DCs, and to a lesser extent of B220$^+$ plasmacytoid DCs. The contribution of CD103$^+$ DCs to asthma protection was further functionally assessed in mice lacking the BATF3 transcription factor, which drives the development of CD8α$^+$ lymphoid tissue-resident DC lineages and of the closely related CD103$^+$CD11b$^-$ DC lineages in various tissues including the lung, intestine and skin (18). We were able to confirm that CD103$^+$CD11b$^-$ DCs are completely absent from the lungs of BATF3$^{-/-}$ mice, whereas all other examined subsets are present in normal numbers. BATF3$^{-/-}$ animals were equally susceptible to allergen-induced asthma as wild-type mice;

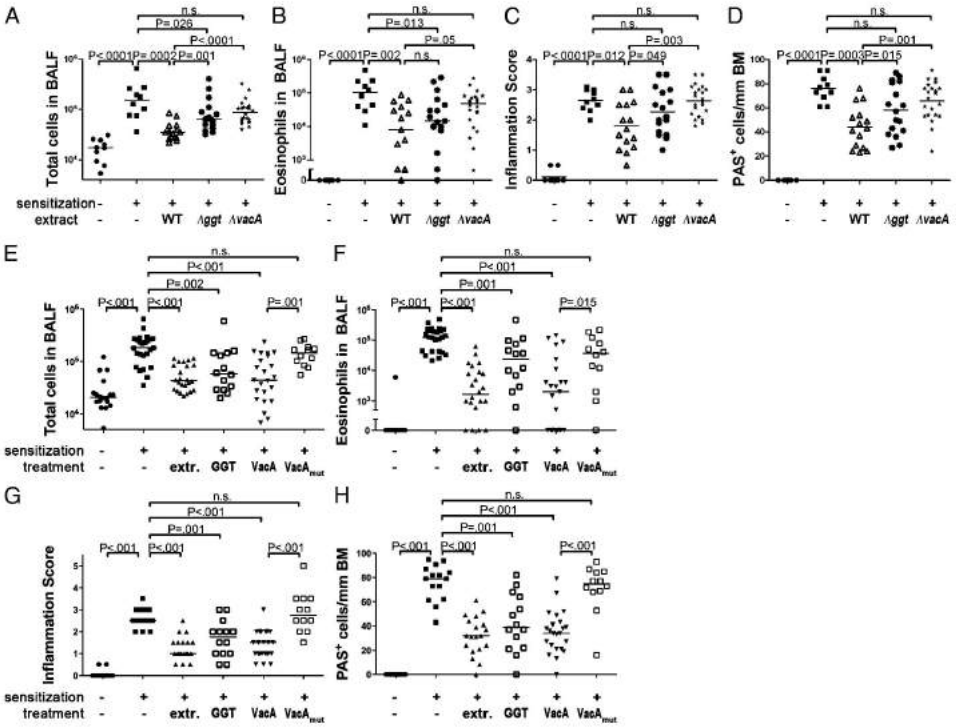

Fig. 4. *H. pylori* GGT and VacA are required and sufficient for protection against asthma. (*A–D*) Groups of mice were treated as described in Fig. 1 with *H. pylori* extract generated from either wild-type (WT) or Δ*ggt* or Δ*vacA* mutant bacteria, and were subjected to ovalbumin sensitization and challenge. (*E–H*) Mice were i.p. injected weekly with 25 μg per dose of either recombinant GGT or purified wild-type or mutant (Δ6–27) VacA starting on day 7 of age until the second sensitization. Total cells (*A* and *E*) and eosinophils (*B* and *F*) contained in 1 mL of BALF. Tissue inflammation (*C* and *G*) and goblet cell metaplasia (*D* and *H*).

however, neither regular extract treatment nor *H. pylori* colonization had any detectable beneficial effect on the examined hallmarks of asthma in this strain, indicating that BATF3-dependent DCs are strictly required for protection in both scenarios. We further found that lymph node-derived DCs from BATF3$^{-/-}$ mice fail to produce IL-10 upon treatment with *H. pylori* extract ex vivo. This observation is well in line with the requirement for IL-10 signaling proficiency and, more specifically, for the DC-intrinsic production of IL-10 for optimal *H. pylori*-mediated protection against allergic asthma. We conclude from the combined results that BATF3-dependent DC lineages suppress pulmonary allergen-specific immune responses by production of IL-10; in contrast, Tregs are not critically required for *H. pylori* extract-mediated protection, as their depletion fails to abrogate protection.

Another cytokine known to be induced by *H. pylori*, IL-18 (22), also turned out to be absolutely essential for asthma prevention in the course of our studies. IL-18 is produced upon inflammasome activation by *H. pylori* in a variety of cell types, including DCs, and promotes Treg differentiation and *H. pylori*-specific tolerance in vitro and in vivo (10, 22). Whether the DC-intrinsic production of IL-18 is required for *H. pylori* (extract)-mediated protection against asthma remains to be addressed with suitable mouse strains.

Our data further show that two *H. pylori* determinants, GGT and VacA, are required for extract-mediated protection and can be administered in purified form to prevent allergic asthma. These findings are in line with earlier reports showing that both factors have a critical role in *H. pylori* persistence and immune modulation. Mutants lacking the *ggt* gene are incapable of colonizing mice persistently (19, 23), and this phenotype has been attributed to DC tolerization by GGT in vitro and in vivo (19). Similarly, a *vacA* gene deletion mutant fails to tolerize DCs and to induce Tregs in vivo, and is therefore effectively controlled or even cleared upon onset of an adaptive immune response (19). The fact that a mutant form of VacA lacking an amino-terminal hydrophobic region of three tandem GXXXG motifs fails to protect against asthma when administered to mice in purified form suggests that membrane insertion by VacA is required for its immunomodulatory effects. The exact mechanism and relevant target cell types of VacA in vivo remain to be elucidated in detail. Taken together, our data demonstrate, to our knowledge for the first time, that the immunomodulatory properties of a very common infectious agent in humans, *H. pylori*, can be exploited for therapeutic purposes in an allergy model and lend

support to *H. pylori*-specific tolerization as a viable strategy for asthma prevention in high-risk individuals.

Materials and Methods

Animal Experimentation. C57BL/6, BATF3$^{-/-}$, IL-18R$^{-/-}$, and CD11c-Cre.IL-10fl/fl mice were orally infected with *H. pylori* PMSS1 as described (16) or received either once-weekly oral or i.p. doses of 200 μg of extract of *H. pylori* wild-type PMSS1, PMSS1Δ*ggt* or PMSS1Δ*vacA* (19), *Salmonella typhimurium*, or *E. coli* or once-weekly i.p. doses of 25 μg of recombinant GGT, or of s1m1 type VacA (wild type or Δ6–27, ref. 20) purified from *H. pylori* strain 60190. Mice were sensitized by i.p. injection of 20 μg of ovalbumin (Sigma-Aldrich) emulsified in 2.25 mg of aluminum hydroxide (Alum Imject; Pierce) at 8 and 10 wk of age and challenged with 1% aerosolized ovalbumin using an ultrasonic nebulizer (NE-U17; Omron) for 20 min daily on days 31, 32, and 33 after initial sensitization. Airway resistance measurements were performed on anesthetized, intubated and mechanically ventilated mice (FinePointe Resistance and Compliance System, Buxco Electronics) in response to increasing doses of inhaled metacholine. In vivo blocking of IL-10 signaling and depletion of Tregs was achieved by two i.p. injections of 250 μg of anti-IL-10R antibody (clone 1B1.3A) and anti-CD25 antibody (clone PC-61.5, both BioXCell), respectively, during the challenge phase. CD4$^+$CD25$^+$ Tregs were adoptively transferred as described (9). Lungs were lavaged via the trachea with 1 mL of PBS. Broncho-alveolar lavage fluid (BALF) cells were counted using trypan blue dye exclusion. Differential cell counts of macrophages, lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with the Microscopy Hemacolor-Set (Merck). For lung histopathology, lungs were fixed by inflation and immersion in 10% (vol/vol) formalin and embedded in paraffin. Tissue sections were stained with H&E and periodic acid-Schiff and examined in blinded fashion on a BX40 Olympus microscope. Peribronchial inflammation was scored on a scale from 0 to 4. PAS-positive goblet cells were quantified per 1 mm of basement membrane. All animal experimentation was performed in accordance with federal, cantonal and institutional guidelines, and approved by the Zurich Cantonal Veterinary Authorities (no. 170/2009 to A.M.).

Preparation of *H. pylori* Extract and Purification of GGT and VacA. *H. pylori* was cultured in *Brucella* broth supplemented with 10% FCS, pelleted by centrifugation, and washed once with PBS. Bacteria were subjected to three freeze/thaw cycles and disrupted by three passes through a French pressure cell press (Stansted Fluid Power, Cell Pressure Homogenizer) at 30,000 bar. Cell debris was removed by centrifugation and the supernatant filtered through a 2-μm filter. Protein concentrations were determined by BCA Protein Kit (R&D Systems). *H. pylori* VacA was purified using published procedures (24, 25), with the following slight modifications. *H. pylori* strain 60190 was cultured in sulfite-free *Brucella* broth containing either cholesterol or 0.5% charcoal. After centrifugation of the culture, supernatant proteins were precipitated with a 50% saturated solution of ammonium sulfate. The oligomeric form of VacA was isolated by gel filtration chromatography with a Superose 6 HR 16/50 column in PBS containing 0.02% sodium azide and 1 mM EDTA. *H. pylori* GGT was purified as described (19). Protocols for lung single cell preparation, flow cytometry, cytokine ELISAs, and preparation of murine and human DCs and DC/T-cell cocultures can be found in *SI Materials and Methods*.

Statistics. All statistical analysis was performed using Graph Pad prism 5.0 software. The Mann–Whitney test was used throughout. *P* values <0.05 were considered significant.

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Supporting Information

Engler et al. 10.1073/pnas.1410579111

SI Materials and Methods

Lung Single Cell Preparation, Flow Cytometry, and Th2 Cytokine ELISAs. Lungs were dissected, enzymatically digested with 0.5 mg/mL collagenase type IA (Sigma-Aldrich) and pushed through a 70-μm nylon cell strainer. The antibodies used for staining were anti-MHCII (clone M5/114.15.2), anti-B220 (RA3-6B2), anti-CD11c (clone HL3), anti-CD103 (clone M290), and anti-CD11b (clone M1/70; all BD Pharmingen). FACS analyses were performed on a FACSCanto2 cytometer (BD Biosciences); post-acquisition analysis was done using FlowJo software (Tree Star). Cytokines in lung single-cell cultures restimulated for 72 h with 250 μg/mL ovalbumin were quantified by ELISA (IL-5, BD Pharmingen; IL-13, R&D Systems).

Preparation of Murine and Human DCs and IL-10 ELISA. For generation of murine bone-marrow-derived dendritic cells (BM-DCs), bone marrow isolated from the hind legs of donor mice (BL/6.TLR2$^{-/-}$, BL/6.TLR4$^{-/-}$, BL/6.MyD88$^{-/-}$ mice, all from Jackson Labs) was seeded at 50,000 cells per well in 96-well plates in RPMI/10% (vol/vol) FCS and 4 ng/mL GM-CSF and cultured for 5 d. For the isolation of MLN-DCs, mesenteric lymph nodes were digested in 1 mg/mL collagenase (Sigma-Aldrich) for 30 min at 37 °C with shaking before filtering through a cell strainer (40 μm; BD Biosciences) and immunomagnetic isolation of DCs using mouse-specific CD11c microbeads (Miltenyi Biotec). BM-DCs and MLN-DCs were stimulated with the indicated amounts of *Helicobacter pylori* PMSS1 extract for 16 h, and supernatants were subjected to mIL-10 ELISA (BD Pharmingen). Human monocyte-derived dendritic cells were generated from peripheral blood mononuclear cells. Venous blood was drawn from six healthy volunteers according to protocols approved by the Institutional Review Board of Leiden University Medical Center. Cells were collected after density gradient centrifugation on Ficoll, and CD14^{+} monocytes were positively isolated by magnetic-activated cell sorting (MACS) using CD14 microbeads (Miltenyi Biotec). Cells were cultured in RPMI-1640 (Invitrogen) supplemented with penicillin (100 U/mL, Astellas Pharma), streptomycin (100 μg/mL, Sigma), pyruvate (1 mM, Sigma), glutamate (2 mM, Sigma), 10% FCS, 20 ng/mL human recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF, Invitrogen/Life Technologies), and 0.86 ng/mL human rIL-4 (R&D Systems) for 6 d. On day 3, the medium and the supplements were refreshed. Monocyte-derived DCs were stimulated with *H. pylori* extract for 48 h. Secretion of IL-10 by the DCs in the supernatant was measured by ELISA (Sanquin).

DC/T-Cell Cocultures. For Treg differentiation ex vivo, BM-DC cultures were infected overnight with wild-type *H. pylori* PMSS1 or treated with 25 μg/mL *H. pylori* extract. Bacteria were killed with 200 U penicillin/0.2 mg streptomycin/mL for 6 h before the addition of T cells. CD4^{+}CD25^{-} T cells were prepared from single-cell suspensions of naive C57BL/6 spleens by immunomagnetic sorting (R&D Systems). DCs were cocultured with CD4^{+}CD25^{-} T cells at a ratio of 1:2 (0.5×10^5 DC to 1×10^5 T cells) in RPMI containing 10% FCS, 10 ng/mL rTGF-β (PeproTech), 10 ng/mL rIL-2 (R&D Systems) and 1 μg/mL anti-CD3ε (BD Bioscience). After 72 h of coculture, the cells were stained first for CD4 and CD25 and then, after fixation and permeabilization, for FoxP3 (FoxP3-APC, eBioscience). The percentage of FoxP3^{+} CD4^{+} T cells was assessed by FACS on a Cyan ADP 9 instrument (Beckman Coulter) and analyzed using FlowJo software (TreeStar).

Processing of Gastric Tissue for Plating and Colony Counting. Stomachs were retrieved and dissected longitudinally. For the quantitative assessment of *H. pylori* colonization, a stomach section containing representative amounts of antral and corpus tissue was homogenized in *Brucella* broth and serial dilutions were plated on horse blood plates for colony counting as described (1).

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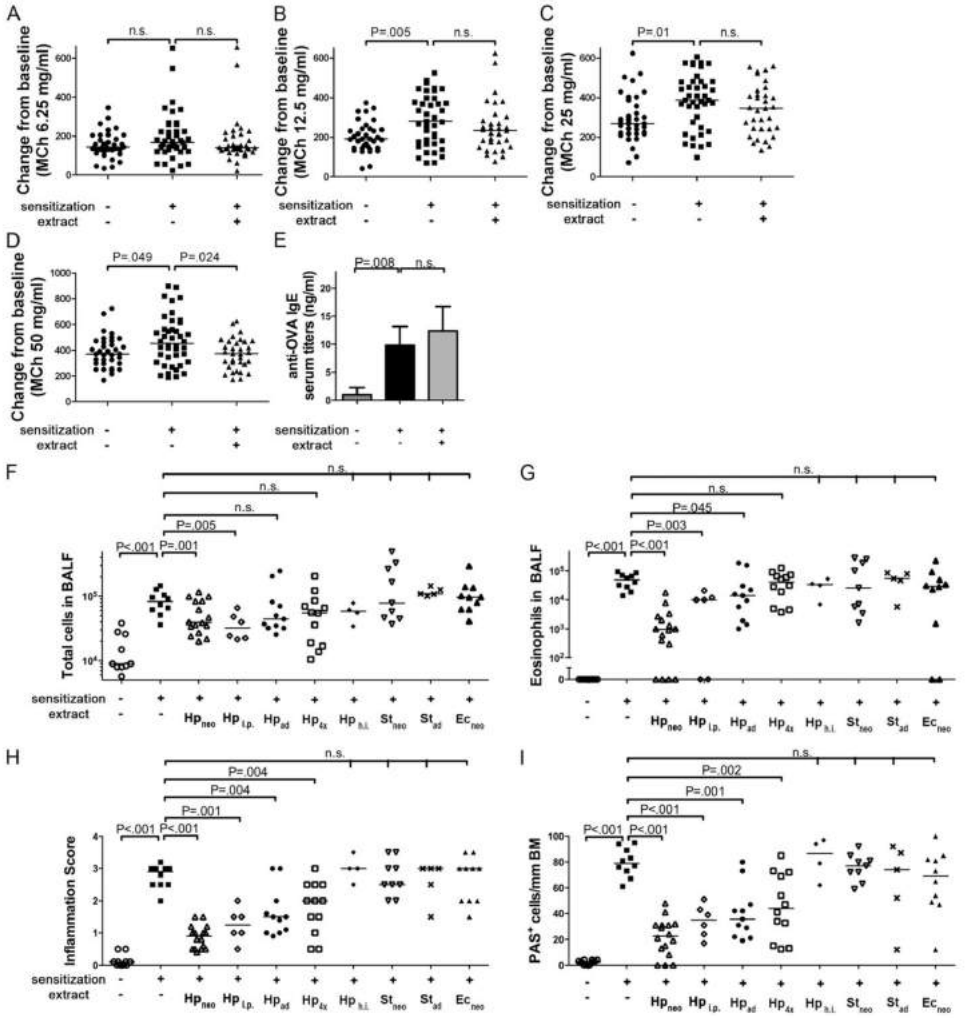

Fig. S1. The protection against asthma conferred by treatment with whole cell extract is specific to *H. pylori*, depends on a heat-sensitive component of the bacteria, and is most efficient when initiated in newborn mice. (*A–E*) Mice were sensitized i.p. with alum-adjuvanted ovalbumin at 8 and 10 wk of age and challenged with aerosolized ovalbumin 2 wk after the second sensitization to induce asthma-like symptoms. Mock-sensitized mice served as negative controls. One group received once-weekly doses of 200 μg of *H. pylori* extract from day 7 of age until the second sensitization. (*A–D*) Airway hyperresponsiveness in response to the indicated increasing doses of methacholine. (*E*) Ovalbumin-specific serum IgE titers of the groups shown in *A–D*. (*F–I*) Mice were treated as described in *A–E*, with the following modifications: Hp_{neo}, extract administered orally from day 7 to the second sensitization, exactly as described above; $Hp_{i.p.}$, extract administered i.p. from day 7 to the second sensitization; Hp_{ad}, extract administered orally to adult mice for 4 wk before the second sensitization; Hp_{4x}, extract administered four times orally in the first 3 wk of life only; $Hp_{h.i.}$, heat-inactivated extract administered orally from day 7 to the second sensitization; $St_{neo/ad}$, Ec_{neo}, *Salmonella typhimurium* and *Escherichia coli* extract administered orally either beginning in the neonatal period or to adults, respectively. (*F* and *G*) Total cells and eosinophils contained in 1 mL of BALF. (*H* and *I*) Tissue inflammation and goblet cell metaplasia. Data points are pooled from two independent studies.

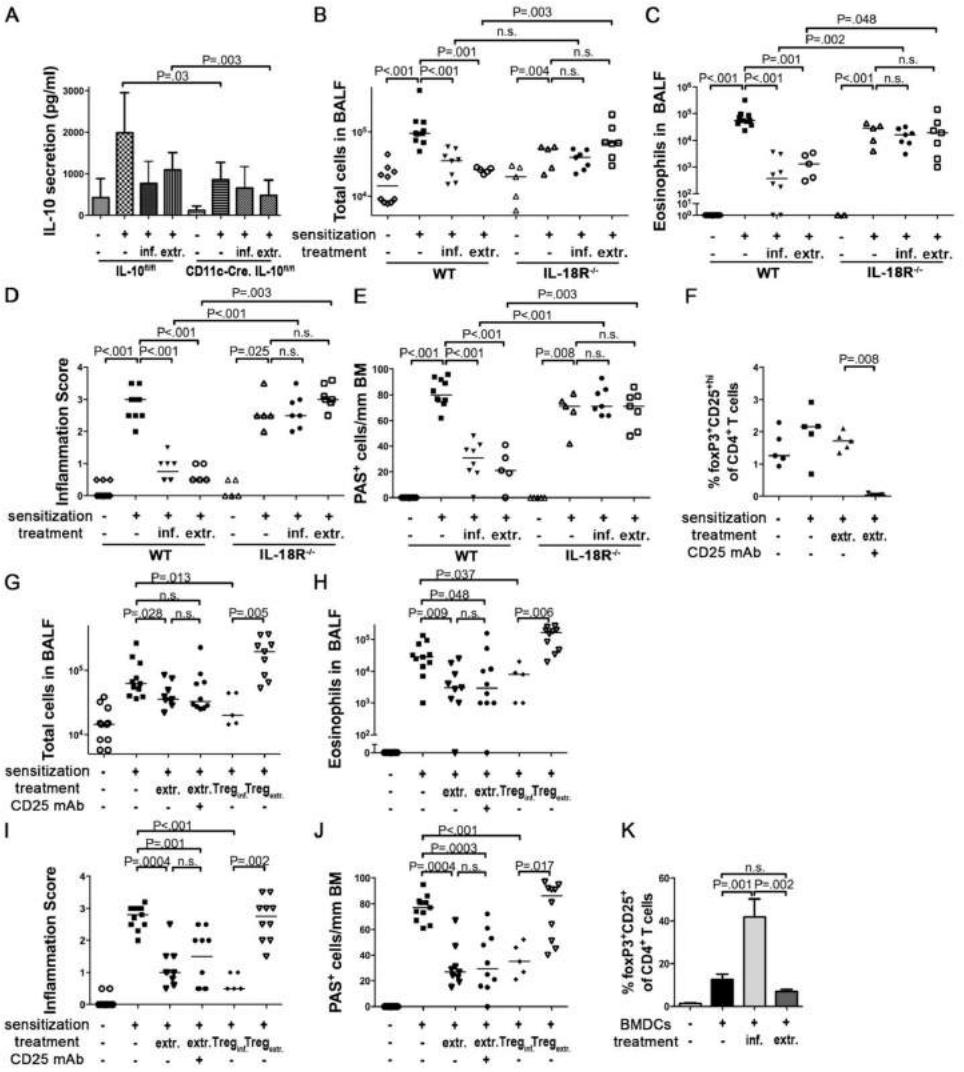

Fig. S2. The protection against asthma conferred by *H. pylori* depends on IL-10 and IL-18, but not on regulatory T cells. (*A*) CD11c-Cre.IL-10$^{fl/fl}$ mice and their IL-10$^{fl/fl}$ littermates were treated with *H. pylori* extract before being subjected to ovalbumin sensitization and challenge as described in Fig. 2 *G–J*. IL-10 secretion by single cell lung preparations restimulated with ovalbumin, as assessed by ELISA. (*B–E*) WT C57BL/6 and IL-18R$^{-/-}$ mice were neonatally infected with *H. pylori* or treated with *H. pylori* extract before being subjected to ovalbumin sensitization and challenge as described in Fig. 1. (*B* and *C*) Total cells and eosinophils contained in 1 mL of BALF. (*D* and *E*) Tissue inflammation and goblet cell metaplasia. (*F–J*) Wild-type C57BL/6 mice were treated with *H. pylori* extract before being subjected to ovalbumin sensitization and challenge. One group received two doses of anti-CD25 antibody during ovalbumin challenge. Additional sensitized and challenged groups received 100,000 immunomagnetically isolated CD4$^+$CD25$^+$ T cells from either neonatally infected donors or extract-treated donors i.v. 2 d before the first ovalbumin challenge. (*F*) Lung infiltration of CD4$^+$CD25$^+$FoxP3$^+$ T cells as assessed in lung single cell preparations of individual mice. (*G* and *H*) Total cells and eosinophils contained in 1 mL of BALF. (*I* and *J*) Tissue inflammation and goblet cell metaplasia. (*K*) BM-DCs were either infected overnight with live *H. pylori* or treated with 25 μg/mL *H. pylori* extract. Bacteria were killed with antibiotics before the addition of splenic CD4$^+$CD25$^-$ T cells at a DC:T-cell ratio of 1:2. DC/T-cell cocultures were supplemented with rTGF-β, rIL-2and anti-CD3ε. After 72 h of coculture, the cells were stained for CD4, CD25, and FoxP3 and the fraction of FoxP3$^+$ CD4$^+$ T cells was assessed by FACS.

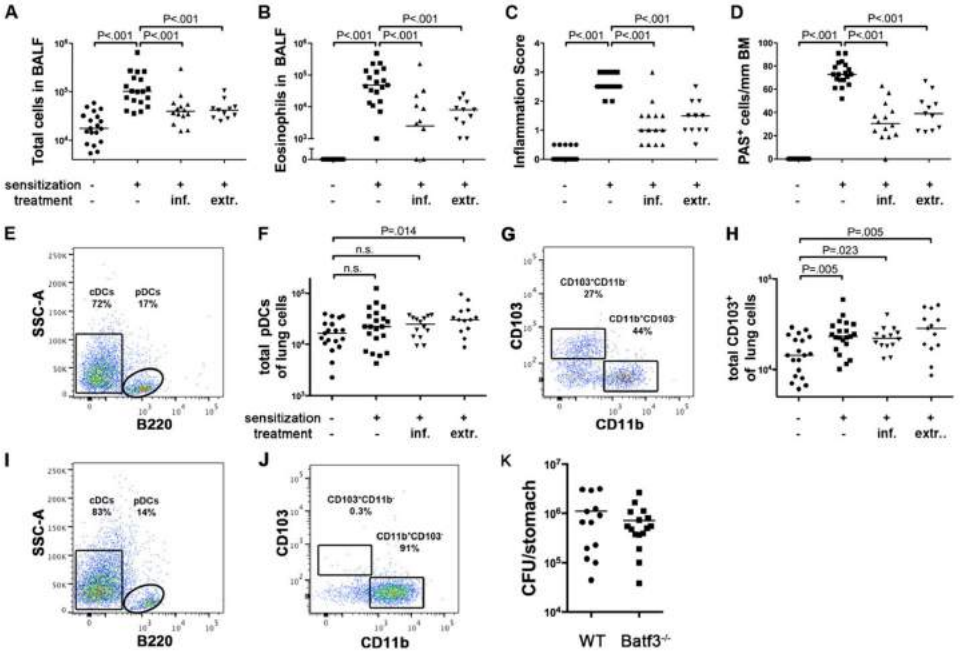

Fig. S3. CD103$^+$ conventional DCs accumulate in the lungs of *H. pylori*-infected and extract-treated wild-type mice, but not basic leucine zipper ATF-like 3 (BATF3)$^{-/-}$ mice. (*A–D*) Total cells and eosinophils contained in 1 mL of BALF, as well as tissue inflammation and goblet cell metaplasia as assessed on H&E and PAS-stained tissue sections, of all mice for which lung DC populations are shown in Fig. 3 *A–G*. (*E*) Plasmacytoid DCs (pDCs) and conventional DCs (cDCs) differentially express B220; a representative scatter plot is shown for all CD11c$^+$ MHCII$^+$ DCs of an extract-treated mouse. (*F*) Total numbers of B220$^+$ pDCs infiltrating the lungs of the mice shown in Fig. 3 *A–G*. (*G*) Two distinct cDC lineages can be discriminated in the lung based on CD103 and CD11b expression; a representative scatter plot is shown for an extract-treated mouse (gated on CD11c$^+$ MHCII$^+$ B220$^-$ DCs). (*H*) Total CD103$^+$ cDC infiltration into the lungs of the mice shown in Fig. 3 *A–G*. (*I* and *J*) BATF3$^{-/-}$ mice lack CD103$^+$ cDCs, but retain CD11b$^+$ cDCs and normal frequencies of pDCs. (*K*) *H. pylori* colonization of neonatally infected WT C57BL/6 and BATF3$^{-/-}$ mice as determined by plating of gastric mucosal homogenates on horse blood plates and colony counting.

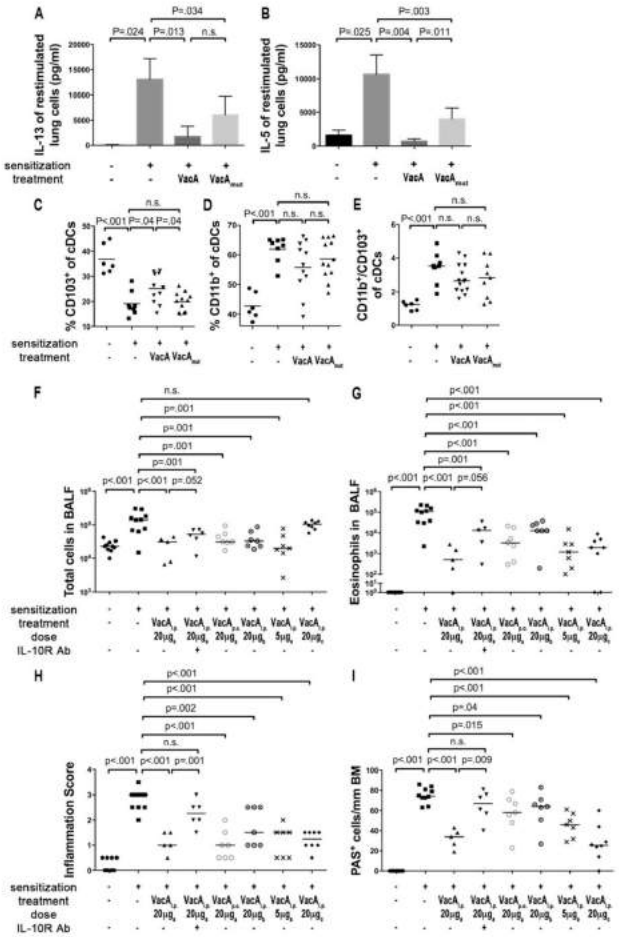

Fig. S4. VacA protects against allergic asthma when administered intraperitoneally or intragastrically. (*A–E*) Mice were i.p. injected weekly with 25 μg per dose of either recombinant GGT or purified wild-type or mutant (Δ6–27) VacA starting on day 7 of age until the second sensitization as described in Fig. 4 *E–H* (a subset of the mice of Fig. 4 *E–H* is shown here). (*A* and *B*) IL-13 and IL-5 secretion, as assessed by ELISA, of single cell lung preparations restimulated with ovalbumin. (*C* and *D*) Frequencies of $CD103^+$ and $CD11b^+$ cells among all $CD11c^+MHC^+B220^-$ cDCs infiltrating the lungs of the indicated groups of mice. (*E*) Ratios of $CD11b^+$ and $CD103^+$ cDCs as calculated per mouse. (*F* and *G*) Purified wild-type VacA was either administered intraperitoneally (i.p.) or intragastrically (p.o.) as indicated. Mice received either 5 μg or 20 μg of VacA (as indicated), either from age day 7 onwards until 2 wk before challenge (as indicated by subscript "a") or three doses only (delivered in weeks 1, 2, and 3 of life, denoted by subscript "b") or as adults (denoted by subscript "c"). Two doses of IL-10R blocking antibody were administered during challenge where indicated. (*F* and *G*) Total cells and eosinophils contained in 1 mL of BALF. (*H* and *I*) Tissue inflammation and goblet cell metaplasia. Three doses of VacA delivered before weaning were insufficient to provide full protection ("b"). Treatment of adults was almost as protective as neonatal-onset treatment (compare "c" and "a"; note that the total cell count in the adult-treated group is inconsistent with the other three readouts for reasons that are not clear); the 5-μg and 20-μg doses provided similar levels of protection; blocking IL-10 signaling abrogated protection.

3.2 *Helicobacter pylori* and its secreted immunomodulator VacA protect against anaphylaxis in experimental models of food allergy

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Contribution: I contributed to the study concept and design, conducted all animal experiments, performed analyses and interpretation of data, and wrote parts of the manuscript.

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ORIGINAL ARTICLE

Experimental Models of Allergic Disease

WILEY

Helicobacter pylori and its secreted immunomodulator VacA protect against anaphylaxis in experimental models of food allergy

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Summary

Background: Food allergy is an increasingly common health problem in Western populations. Epidemiological studies have suggested both positive and negative associations between food allergy and infection with the gastric bacterium *Helicobacter pylori*.

Objective: The objective of this work was to investigate whether experimental infection with *H. pylori*, or prophylactic treatment with *H. pylori*-derived immunomodulatory molecules, affects the onset and severity of food allergy, either positively or negatively.

Methods: We infected neonatal C57BL/6 or C3H mice with *H. pylori* or treated animals with *H. pylori* components (bacterial lysate or the immunomodulator VacA) and subsequently subjected them to four different protocols for food allergy induction, using either ovalbumin or peanut extract as allergens for sensitization and challenge. Readouts included anaphylaxis scoring, quantification of allergen-specific serum IgE and IgG1 and of the mast cell protease MCPT1, as well as splenic T-helper-2 cell-derived cytokine production. Mesenteric lymph node $CD4^+FoxP3^+$ regulatory T cells were subjected to flow cytometric quantification and sorting followed by qRT-PCR, and to DNA methylation analyses of the Treg-specific demethylated region (TSDR) within the *FOXP3* locus.

Results: Mice that had been infected with *H. pylori* or treated with *H. pylori*-derived immunomodulators showed reduced anaphylaxis upon allergen sensitization and challenge, irrespective of the allergen used. Most of the immunologic assays confirmed a protective effect of *H. pylori*. $CD4^+FoxP3^+$ T cells were more abundant in protected mice and exhibited a stable Treg phenotype characterized by *FOXP3* TSDR demethylation.

Conclusions and Clinical Relevance: *Helicobacter pylori* confers protection against the anaphylaxis associated with ovalbumin and peanut allergy and affects the epigenome of T cells, thereby promoting stable Treg differentiation and functionality. Prophylactic treatment with *H. pylori*-derived immunomodulators appears to be a promising strategy for food allergy prevention.

KEYWORDS
anaphylaxis, bacterial immunomodulators, epigenetics, food allergy

1 | INTRODUCTION

Food allergy is an increasingly common condition in Western countries as well as Asia, affecting ~6-8% of children and ~4% of adults.[1,2] It is caused by inappropriate T cell-driven immune responses to harmless food components of milk, eggs, peanuts, tree nuts, seafood, shellfish, soya, wheat (the "big eight") and other foods. Food allergy often co-occurs with other atopic diseases such as allergic rhinitis and conjunctivitis, eczema and allergic asthma and is believed to result mechanistically from the breakdown of the T cell suppressive mechanisms conferred by oral immune tolerance. Symptoms of food allergy usually occur with a fast onset (from seconds to 1 hour) and include rashes, hives, swelling and itching of parts or the whole face, wheezing, diarrhoea, nausea and, in severe cases, anaphylaxis. The mainstay of treatment is the strict avoidance of food allergens; accidental intake requires epinephrine injections in severe and antihistamines and steroids in mild cases. The benefits of allergen immunotherapy for food allergies are currently unclear, and the procedures are thus not (yet) recommended.

The hygiene[3] and disappearing microbiota[4] hypotheses postulate that the reduced exposure to environmental and pathogenic microbes early in life and/or the loss of ancestral indigenous microbes colonizing various niches of the human body contribute to an increased allergy risk. Both hypotheses have served to explain the dramatic increase in the prevalence of food allergy in Western countries. The paradigmatic example of a microbe that is increasingly lost from human populations is *H. pylori*. Chronic infection with this gastric colonizer on the one hand represents an important risk factor for the development of gastritis, ulcers and gastric cancer[5,6] but on the other hand appears to confer protection against oesophageal disorders such as gastro-oesophageal reflux disease and oesophageal cancer, as well as asthma, allergy and inflammatory bowel diseases.[7,8] Whereas the inverse association of *H. pylori* with allergic asthma, rhinitis and eczema is well supported by large epidemiological studies and meta-analyses,[9-12] the effects of *H. pylori* positivity on the risk of developing food allergy are controversial. Few reports are available, and the existing studies suffer from small sample sizes, heterogeneous populations and non-standardized methodologies.[13] Several studies have found a positive association of *H. pylori*, especially of strains harbouring the virulence factor CagA, with food allergy in children[14,15] or adults.[16] Other studies have found no association,[17] or a negative association.[12] The latter study reported both a decreased seroprevalence of *H. pylori* in food allergy patients relative to controls (33% vs 40% as determined by urea breath test and serology) as well as a reduced production of allergic mediators (such as eosinophilic cationic protein and mast cell tryptase) in *H. pylori*-positive relative to *H. pylori*-negative food allergy patients.[12] Mechanistically, positive associations with food allergy were explained by a breakdown of the epithelial barrier to food allergens due to the chronic inflammation of the *H. pylori*-infected gastric mucosa, whereas negative associations were attributed to the immunomodulatory activity of *H. pylori* on the activation and polarization of T cell responses.

We have previously examined the role of *H. pylori* in various experimental models of allergic asthma and have consistently detected a strong protective effect, especially of neonatal exposure to the bacteria, on the development of allergic asthma in response to various allergens.[18-20] Given the controversial results from observational studies in humans with food allergy, the strongly increasing prevalence of food allergy in children and adults, and the robust effects observed in our allergic asthma models, we asked whether experimental *H. pylori* infection would alleviate the clinical and immunological symptoms of food allergy induced by two different common food allergens. We used recombinant ovalbumin and peanut extract, administered via various routes, to trigger anaphylaxis symptoms in two strains of mice. When administered intraperitoneally for the purpose of allergen challenge, peanut extract was superior to ovalbumin in inducing swelling and oedema of the mucosal surfaces of the face, as well as a variety of systemic parameters related to Th2, B cell and mast cell activity. *H. pylori* infection and *H. pylori* extract treatment, and also the administration of several doses of purified VacA, reduced clinical symptoms of food allergy in all or some of the four examined models and decreased Th2 cytokine production, mast cell protease secretion and allergen-specific serum IgG1 levels. The same treatments could be shown to promote Treg numbers, regulatory activity and stability by demethylating the TSDR of the *FOXP3* locus in regulatory T cells (Tregs) of mesenteric lymph nodes. Our results thus suggest that *H. pylori* down-modulates immune responses to common food allergens by affecting the epigenome of Tregs and promoting their stable lineage differentiation, thereby conferring protection against various clinical and immunological hallmarks of food allergy.

2 | METHODS

2.1 | Animal experimentation

C57BL/6 and C3H mice were purchased from Janvier and included in allergy experiments at 5-7 weeks of age. For the induction of ovalbumin (OVA)-induced food allergy, C57BL/6 mice were sensitized twice i.p. with 50 μg OVA (Sigma A5503-5G, Buchs, Switzerland) emulsified in aluminium hydroxide (Alum Imject, Thermo Scientific 77161, Reinach, Switzerland) on days 0 and 14, followed by challenge via oral gavage on days 28, 29, 30 and 31 with 60 mg OVA. Symptoms were scored after each of the first three challenges; mice were killed by CO_2 inhalation 30-45 minutes after the last challenge; and blood and tissue samples were collected. For the induction of peanut extract (PE)-induced food allergy, C57BL/6 mice were sensitized orally once a week for 4 weeks with 2 mg PE adjuvanted with 20 μg cholera toxin (List Biologicals 101B, Campbell, California, USA) followed by either four oral challenges on four consecutive days with 10 mg PE (in this model, symptoms were scored after the first three challenges) or two i.p. challenges (with a 2-day break) with 1 mg PE (in that case, symptoms were scored after the first challenge). Scoring was done for 40 minutes beginning right after challenge, with scores indicating the following: 0, no sign of reaction; 1,

repetitive scratching and rubbing around the nose/mouth and head, ear canal digging with hind legs; 2, decreased activity with an increased respiratory rate, pilar erecti and/or puffing around the eyes and/or mouth; 3, laboured respiration and cyanosis around the mouth and tail and/or periods of motionless for more than 1 minutes; lying prone on stomach; 4, slight or no activity after prodding/whisker stimuli or tremors and convulsion; 5, death. In the oral challenge models, cumulative scores were calculated by adding up three individual scores per mouse. C3H mice were sensitized orally once a week for 5 weeks with 2 mg PE adjuvanted with 20 μg cholera toxin followed by two oral challenges on 2 consecutive days with 14 mg PE (symptoms were scored after the first challenge). The processing and analysis of animal tissues is described below; MCPT1 in serum was quantified by ELISA (88-7503-88, Thermo Scientific) according to the manufacturer's instructions. All animal experimentation was reviewed and approved by the Zurich Cantonal Veterinary Office (licence 170/2014 to A.M.). The *H. pylori* strain PMSS1 was cultured as previously described[21]; mice were infected by oral gavage on days 6 and 7 after birth with 10^7 to 10^8 bacteria. For the production of *H. pylori* extract, bacterial cultures were pelleted, washed with PBS and subjected to three freeze-thaw cycles and homogenization using a pressure cell homogenizer (Stansted SPCH-18). The homogenate was centrifuged at 3000 × *g*, the resulting supernatant was sterile filtered, and the protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific 23227). Oligomeric s1 m1 type VacA was purified from *H. pylori* strain 60 190 as described previously.[22] The dosage of extract and VacA was adjusted to the age of the mice and the application mode: extract i.p. 5-100 μg, extract p.o. 50-200 μg, VacA i.p. 5-20 μg. Mice were treated with VacA or extract once a week. For the production of peanut extract, partially defatted peanut flour (Golden Peanut Company, Alpharetta, Georgia, USA) was extracted overnight in 10 × PBS, solid parts were removed by centrifugation, and extract was concentrated using Amicon Ultracel 3K centrifugal filters (Merck Millipore UFC900324, Schaffhausen, Switzerland). The final protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific 23227).

2.2 | Allergen-specific ELISAs

For OVA-specific IgG1 ELISA, high-affinity plates were coated with 10 μg OVA in carbonate-bicarbonate coating buffer (pH 9.6) overnight at 4°C. After washing and blocking with 2% BSA in PBS, serum samples were diluted in 1% BSA/PBS and added for 2 hours at 37°C. After further washing, the plates were incubated for 1 hour at 37°C with HRP-coupled anti-mouse IgG1 (eBioscience 18-4015-82). Wells were washed again before adding HRP substrate and measuring absorbance in a Spectromax plate reader. OVA-specific IgE was measured using the same procedure as described for IgG1, except that HRP-coupled anti-mouse IgE (GeneTex GTX77227) was used for detection. For PE-specific IgE ELISA, high-affinity plates were coated with 500 μg PE overnight as described above. After washing and blocking with 5% gelatine in PBS at 37°C for 1 hour, serum samples diluted in 2% BSA/PBS were added to the plate for 2 hours at 37°C. After further washing, the plates were incubated with anti-mouse IgE-biotin (BD Biosciences 553419, Allschwil, Switzerland) and washed before avidin-HRP (Thermo Scientific 21130) was added. Wells were washed again before addition of HRP substrate and absorbance detection in a plate reader.

2.3 | Re-stimulation of splenocytes and cytokine ELISAs

Spleens were pushed through a 40-μm cell strainer and washed with PBS prior to red blood cell lysis. Splenocytes were seeded into 96-well plates in RPMI 1640 medium (Gibco 21875-034 plus FCS and Penicillin-Streptomycin) supplemented with 200 μg/mL PE or OVA. After 4 days in culture, supernatants were collected and stored at −20°C until cytokines were quantified by IL-5 (88-7054-88) and IL-13 (88-7137-88) ELISA according to the manufacturer's instructions (eBioscience).

2.4 | Treg-specific demethylated region methylation analysis

Total mesenteric lymph node cells of male C57BL/6 mice were isolated by means of collagenase type IV (Sigma C5138) digestion and pushing through a cell strainer. After fixation, permeabilization and washing, the cells were stained with anti-mouse CD4-FITC (Biolegend 100510, London, UK) and Foxp3-APC antibodies (eBioscience 17-5773-82). $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells were sorted on a FACS Aria. Genomic DNA was isolated from sorted cell subsets using the NucleoSpin® Tissue kit (Macherey-Nagel, Oensingen, Switzerland). An additional step was added to the manufacturer's protocol to remove formaldehyde-induced crosslinking. Briefly, Chelex-100 beads (Bio-Rad, Cressier, Switzerland) were added after the lysis step and incubated at 95°C for 15 minutes in a shaker. Chelex-100 beads were spun down, and the supernatant was transferred to a fresh tube. After addition of an adjusted amount of 100% ethanol, the following purification steps were performed according to the manufacturer's protocol. Genomic DNA was converted with bisulphite using the EZ DNA Methylation Kit (Zymo Research, Benley Cir Tustin, California, USA) according to the manufacturer's instructions. The Treg-specific demethylated region (TSDR) was amplified by PCR and analysed by pyrosequencing on a PSQ96MA (Qiagen, Hilden, Germany) as described recently.[23] Primers for sequencing were (in 5′ to 3′ direction), S1:CCATACAAAACCCAAATTC, S2: ACCCAAA TAAAATAATATAAATACT, S3: ATCTACCCCACAAATTT, S4: AACC AAATTTTTCTACCATT), which cover CpG motifs 3-12 of the TSDR core region.

2.5 | Flow cytometric analysis and cell sorting for qRT-PCR

Total mesenteric lymph node cells of male C57BL/6 mice were isolated by pushing through a cell strainer. After fixation,

permeabilization and washing, the cells were stained with anti-mouse CD4 PerCP/Cy5.5 (Biolegend 116012), anti-mouse CD45 BV650 (Biolegend 103151), anti-mouse TCR β chain PE/Cy7 (Biolegend 109222), anti-mouse CD8α BV510 (Biolegend 100752), anti-mouse Foxp3 BV421 (Biolegend 126419), the fixable viability dye eFluor780 (eBioscience 65-0865-14) and flow cytometrically measured using a LSR II Fortessa instrument followed by a detailed analysis using FlowJo software. For FACS-sorting of regulatory T cells, mesenteric lymph node cells were stained with anti-mouse CD45 BV650 (Biolegend 103151), anti-mouse TCR β chain PE/Cy7 (Biolegend 109222), anti-mouse CD4 BV711 (Biolegend 100550), anti-mouse CD25 PE (Biolegend 101903), fixable viability dye eFluor780 (eBioscience 65-0865-14) and sorted on a FACS Aria. RNA of sorted cells was isolated using the RNeasy Mini Kit (Qiagen 74106), converted into cDNA and subjected to TaqMan Real-Time PCR assay using the primers Mm03024075 (Hprt), Mm00475162 (Foxp3), Mm01178820 (Tgfb1) and Mm01288386 (IL10; all from Thermo Fisher Scientific). Samples were run on a Light Cycler 480 and normalized to the house-keeping gene *Hprt*.

2.6 | Statistical analysis

GraphPad Prism 6 was used for all statistical analyses. In all graphs, each symbol represents an individual animal and horizontal lines indicate medians. The Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout. Stars are used to indicate the level of significance according to the *P*-value: $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

3 | RESULTS

3.1 | Live *Helicobacter pylori* infection or treatment with *H. pylori* whole-cell extract alleviates symptoms in an ovalbumin-induced food allergy model

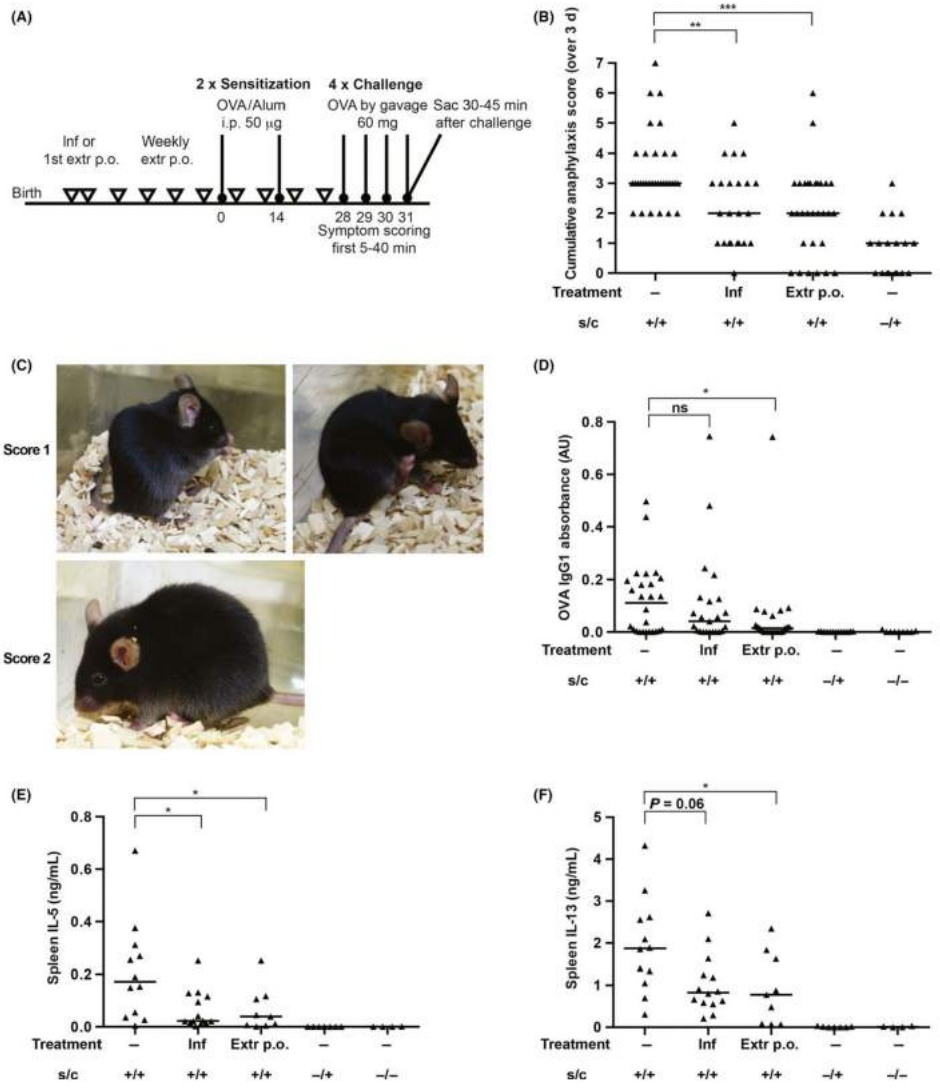
To assess whether infection with *H. pylori* or treatment with whole-cell extract protects against allergic responses to ingested ovalbumin, we either infected neonatal C57BL/6 mice with live *H. pylori* on days 6 and 7 after birth or intragastrically administered weekly doses of whole-cell extract from day 7 onwards. As adults, mice were subjected to intraperitoneal ovalbumin (OVA, adjuvanted with alum) sensitization followed by intragastric OVA challenges (see schematic in Figure 1A). Mice were scored for anaphylactic symptoms such as scratching around the nose, mouth and ears, decreased activity and increased respiratory rate and puffy eyes for 40 minutes after each challenge. At the study end-point, serum levels of OVA-specific IgE and IgG1 antibodies, serum levels of mast cell protease 1 (MCPT1) and the splenic production of the Th2 cytokines IL-5 and IL-13 were quantified by ELISA. Mice that had been sensitized and challenged with OVA (positive controls) developed mild-to-moderate anaphylactic symptoms accompanied by high serum titres of OVA-specific IgE and IgG1 and elevated levels of MCPT1, a systemic marker of mast cell degranulation (Figure 1B-D, Fig. S1A,B). The re-stimulation of splenic single-cell preparations with OVA revealed a clearly elevated production of IL-5 and IL-13 relative to control groups that had not been sensitized (but challenged) or had never been exposed to OVA (Figure 1E,F). In contrast, mice that were infected with *H. pylori* or had received *H. pylori* whole-cell extract were assigned significantly lower anaphylaxis scores, exhibited reduced serum levels of OVA-specific IgG1 and produced lower amounts of splenic Th2 cytokines upon re-stimulation (Figure 1B-F); the assessment of serum MCPT-1 levels confirmed these trends for the extract treatment, but not for the live infection, and serum IgE levels were not reduced by either extract treatment or live infection (Fig. S1A,B). Overall, the results indicate that *H. pylori* infection and extract treatment efficiently reduce anaphylaxis symptoms and most other hallmarks of ovalbumin-specific allergy; the effects on anaphylaxis scores in particular, the most relevant readout from a clinical perspective, are strong and robust.

3.2 | Food allergy symptoms induced by peanut extract are reduced by *Helicobacter pylori* infection and treatment with *H. pylori* extract or the *H. pylori* immunomodulator VacA

We next sought to determine whether these results could be corroborated in additional models of food allergy. As proteins contained in peanuts are among the most common food allergens in Western societies, we opted for peanut-specific sensitization and challenge. Mice received four intragastric weekly doses of cholera toxin-adjuvanted peanut extract (PE) for the purpose of sensitization, followed by intragastric challenge with PE on 4 consecutive days

FIGURE 1 OVA- induced food allergy is ameliorated by neonatal *Helicobacter pylori* infection or weekly extract treatment. (A) Schematic of OVA-induced food allergy and *H. pylori*-specific interventions. Mice were sensitized twice intraperitoneally (i.p.) with 50 μg of alum-adjuvanted OVA and orally challenged 4 wks after the first sensitization on 4 consecutive days with 60 mg OVA (s/c; positive controls). Negative control animals were challenged but not sensitized, or neither sensitized nor challenged. One group of mice was neonatally infected on day 6 and 7 after birth with the *H. pylori* strain PMSS1 (inf), and another group was treated weekly by oral gavage with 100-200 μg (adjusted to body weight) whole-cell *H. pylori* extract starting on day 6 or 7 after birth (extr p.o.). (B) Cumulative anaphylaxis score assigned upon challenge (sum of three scores). s/c, sensitization and challenge. (C) Representative pictures showing mice with a score of 1 (repetitive scratching around nose and head, and hind-leg-ear-digging) and a score of 2 (puffing around eyes, pilar erecti, decreased activity). (D) Serum OVA-specific IgG1, as determined by ELISA. (E,F) Cytokine production, as determined by IL-5 and IL-13 ELISA, of splenocytes that had been re-stimulated in vitro with OVA for 4 d. In B,D,E and F, each symbol represents one mouse. Graphs show pooled data from 5 (B), 4 (D) and 2 (E,F) experiments. Horizontal lines indicate medians; the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout for the calculation of *P*-values. $*P < .05$, $**P < .01$, $***P < .001$

beginning 1 week after the last sensitization (Figure 2A). Neonatally infected mice and mice treated with either *H. pylori* extract or the purified *H. pylori* immunomodulator VacA were sensitized and challenged alongside a group of positive controls that received no intervention whatsoever. The food allergy symptoms elicited by intragastric sensitization and challenge with peanut extract were relatively mild and comparable to symptoms induced with OVA (Figure 2B-D). Similar to the OVA model, regular treatment with extract or the purified immunomodulator VacA effectively reduced the anaphylaxis scores, but failed to affect peanut-specific serum IgE levels; live infection had no significant effect on any of the parameters but showed a trend towards lower anaphylaxis scores (Figure 2B,C). MCPT serum levels were modestly reduced by all three interventions (Figure 2D), albeit not significantly. In summary, we



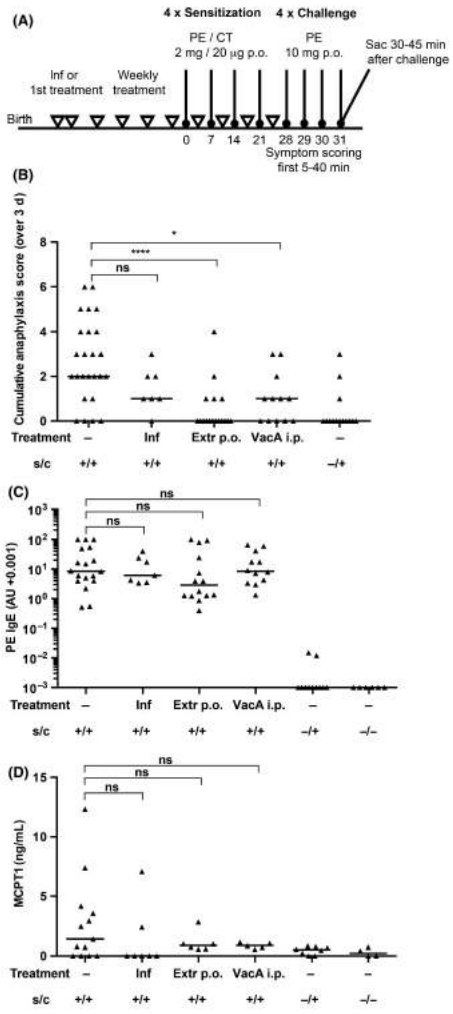


FIGURE 2 Amelioration of allergic symptoms in an oral challenge peanut allergy model upon *Helicobacter pylori*-specific treatments. (A) Schematic of PE-induced food allergy and *H. pylori* interventions. Mice were sensitized four times by oral gavage (p.o.) with 2 mg of peanut extract (PE) adjuvanted with 20 µg cholera toxin (CT), followed 4 wks later by oral challenges on 4 consecutive days with 60 mg OVA (positive controls). Negative control animals were challenged but not sensitized, or neither sensitized nor challenged. One group of mice was neonatally infected on days 6 and 7 after birth with the *H. pylori* strain PMSS1 (inf), and another group was treated weekly by oral gavage with 100-200 µg (adjusted to body weight) whole-cell *H. pylori* extract starting on days 6 or 7 after birth (extr p.o.). A final group received once-weekly increasing doses (adjusted to body weight) of 5-20 µg purified VacA (VacA i.p.) starting on days 6 or 7 after birth. (B) Cumulative anaphylaxis score assigned upon challenge (sum of three scores). (C,D) Serum PE-specific IgE and MCPT1 levels, as determined by ELISA. In B-D, each symbol represents one mouse. Graphs show pooled data from 4 (B), 3 (C) and 2 (D) experiments. Horizontal lines indicate medians; the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout for the calculation of *P*-values.*$P < .05$, ****$P < .0001$

observe in two complementary food allergy models that *H. pylori*-based prophylactic interventions reduce clinically relevant symptoms of food allergy and modulate immunologic correlates of the disease.

3.3 | Severe anaphylaxis induced by peanut extract is alleviated by *Helicobacter pylori*

Both above-described models of food allergy produce relatively mild symptoms. Mice rarely develop systemic symptoms such as decreased activity or effects on respiration rates. To mimic more severe anaphylactic reactions, which can be life-threatening in peanut-allergic children, we devised a model that produced significantly higher anaphylaxis scores, accompanied by higher serum MCPT levels and stronger cytokine production by re-stimulated splenocytes relative to the other two models. In this model, mice were sensitized orally by four CT-adjuvanted doses of peanut extract followed by two intraperitoneal challenges with the same extract (Figure 3A). Most mice of the positive control group were assigned anaphylaxis scores of three (defined as laboured respiration and cyanosis around the mouth and tail and/or periods of motionless for more than 1 minutes, lying prone on stomach) or four (slight or no activity after prodding/whisker stimuli or tremors and convulsion; Figure 3B,C) already after the first challenge dose. Anaphylaxis scores were strongly reduced by treatment with VacA, but not so much by the other examined interventions (Figure 3B); moreover, the Th2 cytokine production of re-stimulated splenocytes (Figure 3D,E) and MCPT and IgE levels measured in serum showed only modest trends (Fig. S2A,B). Finally, we switched to a fourth model of food allergy, this time using particularly anaphylaxis-sensitive C3H mice.[24] These mice exhibited anaphylaxis scores of up to three upon oral sensitization and challenge, and thus indeed proved to be more susceptible to peanut extract than C57BL/6 mice also in our hands (Fig. S3A-C). Regular *H. pylori* extract treatment had modest, but not statistically significant, effects on anaphylaxis scores, serum MCPT levels and spleen weights (Fig. S3B-E). Treatment with VacA had similar effects, but too few mice were analysed to draw definitive conclusions (Fig. S3B-E). In summary, the severe anaphylaxis induced by the intraperitoneal administration of allergen to sensitized C57BL/6 mice, or the oral challenge of C3H mice, can be reduced somewhat by prior infection with *H. pylori*, or the regular exposure to *H. pylori* extract or VacA.

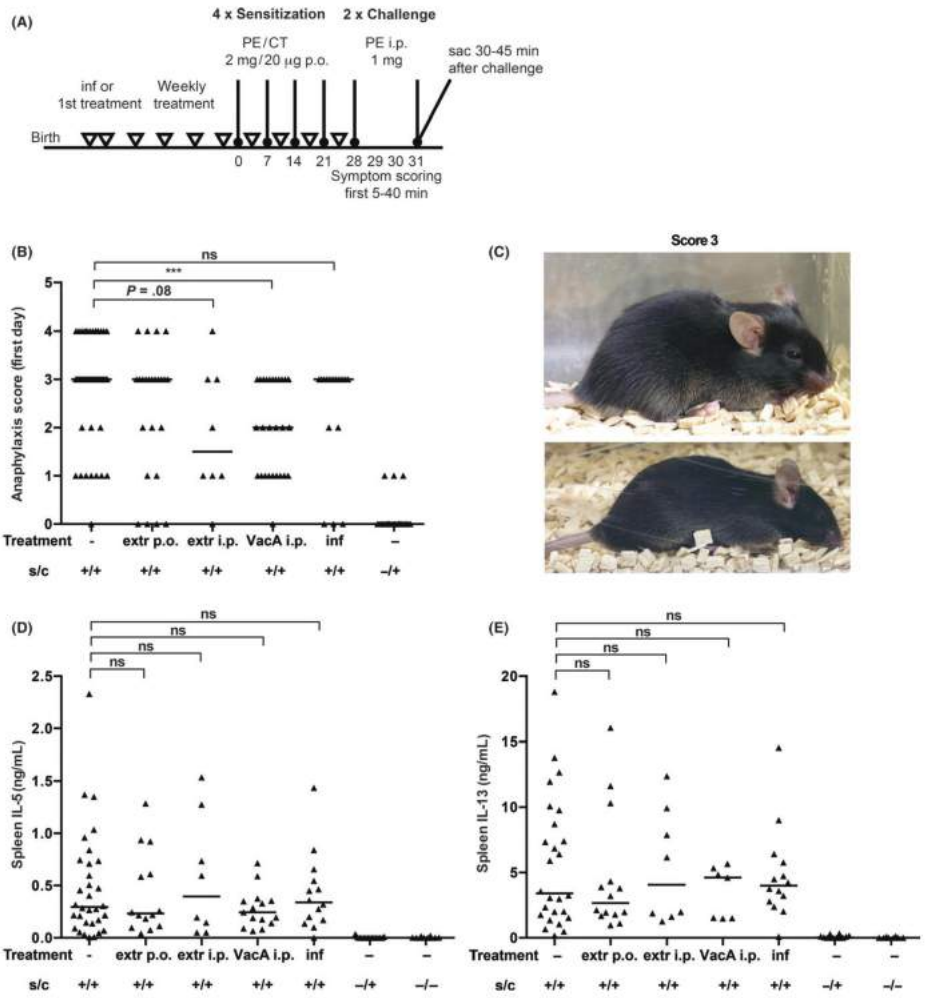
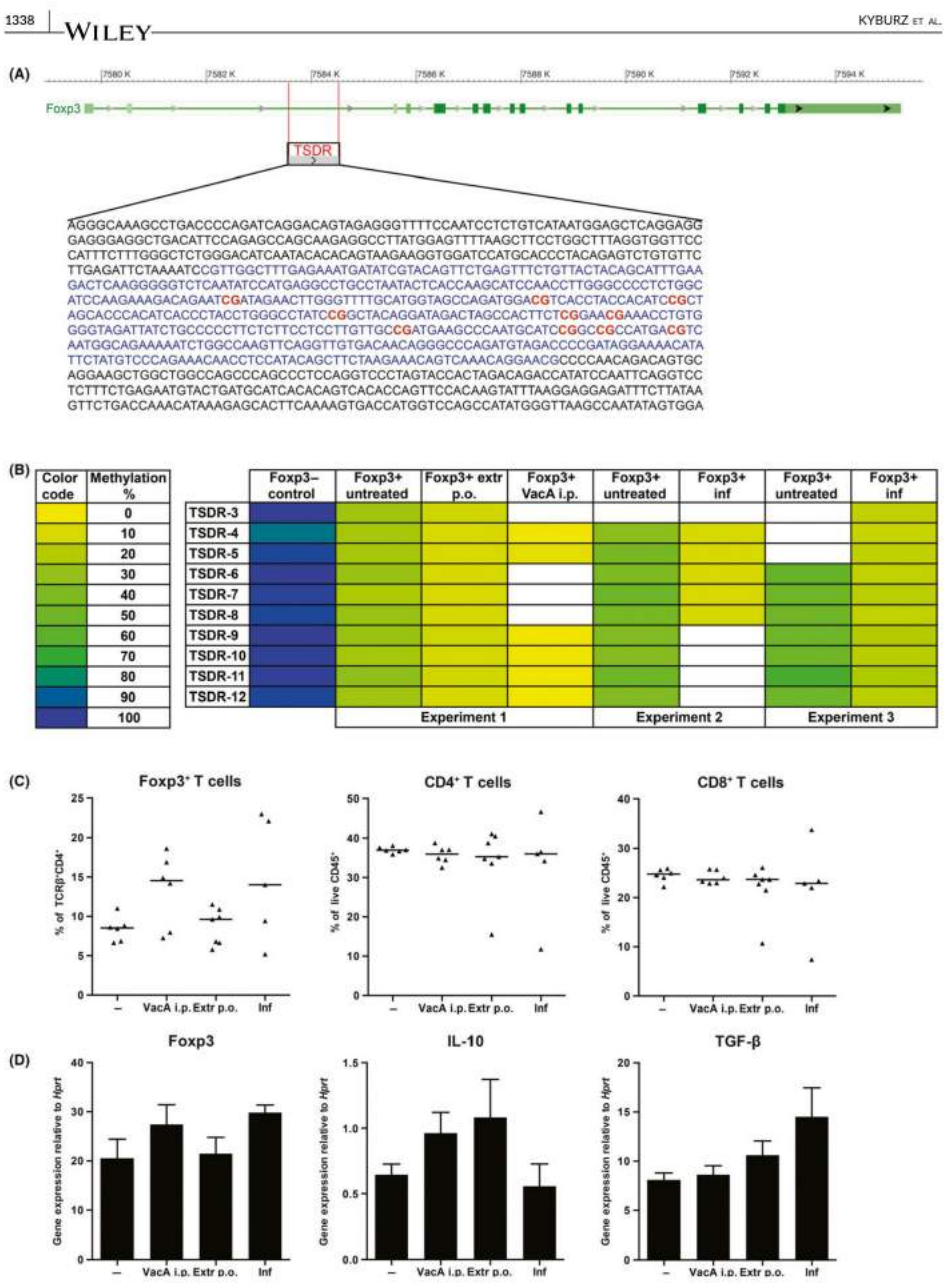


FIGURE 3 Systemically induced peanut food allergy is reduced upon neonatal *Helicobacter pylori* infection or weekly treatment with extract or VacA. (A) Schematic of PE-induced food allergy and *H. pylori* interventions. Mice were sensitized four times by oral gavage (p.o.) with 2 mg of peanut extract (PE) adjuvanted with 20 μg cholera toxin (CT), followed 1 wk later by two i.p. challenges (with a 3-d interval) with 1 mg PE (positive controls). Negative control animals were challenged but not sensitized, or neither sensitized nor challenged. One group of mice was neonatally infected on day 6 and 7 after birth with the *H. pylori* strain PMSS1 (inf), and another group was treated weekly by oral gavage or i.p. with whole-cell *H. pylori* extract starting on day 6 or 7 after birth (extr p.o./i.p.). A final group received weekly increasing doses (adjusted to body weight) of 5-20 μg purified VacA (VacA i.p.) starting on day 6 or 7 after birth. (B) Anaphylaxis score assigned at first challenge. (C) Representative pictures showing mice with a score of 3 (lying prone on stomach for more than 1 min). (D,E) Cytokine production, as determined by IL-5 and IL-13 ELISA, of splenocytes that had been re-stimulated in vitro with PE for 4 d. In B,D and E, each symbol represents one mouse. Graphs show pooled data from 8 (B) and 6 (D,E) experiments. Horizontal lines indicate medians; the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout for the calculation of *P*-values. ***$P < .001$


1338 WILEY KYBURZ ET AL.
(A)
7580 K
7582 K
7584 K
7586 K
7588 K
7590 K
7592 K
7594 K
Foxp3
TSDR
AGGGCAAAGCCTGACCCCAGATCAGGACAGTAGAGGGTTTTCCAATCCTCTGTCATAATGGAGCTCAGGAGG
GAGGGAGGCTGACATTCCAGAGCCAGCAAGAGGCCTTATGGAGTTTTAAGCTTCCTGGCTTTAGGTGGTTCC
CATTTCTTTGGGCTCTGGGACATCAATACACACAGTAAGAAGGTGGATCCATGCACCCTACAGAGTCTGTGTTC
TTGAGATTCTAAAATCCGTTGGCTTTGAGAAATGATATCGTACAGTTCTGAGTTTCTGTTACTACAGCATTTGAA
GACTCAAGGGGGTCTCAATATCCATGAGGCCTGCCTAATACTCACCAAGCATCCAACCTTGGGCCCCTCTGGC
ATCCAAGAAAGACAGAATCGATAGAACTTGGGTTTTGCATGGTAGCCAGATGGACGTCACCTACCACATCCGCT
AGCACCCACATCACCCTACCTGGGCCTATCCGGCTACAGGATAGACTAGCCCACTTCTCGGAACGAAACCTGTG
GGGTAGATTATCTGCCCCCTTCTCTTCCTCCTTGTTGCCGATGAAGCCCAATGCATCCGGCCGCCATGACGTC
AATGGCAGAAAAATCTGGCCAAGTTCAGGTTGTGACAACAGGGCCCAGATGTAGACCCCGATAGGAAAACATA
TTCTATGTCCCAGAAACAACCTCCATACAGCTTCTAAGAAACAGTCAAACAGGAACGCCCCAACAGACAGTGC
AGGAAGCTGGCTGGCCAGCCCAGCCCTCCAGGTCCCTAGTACCACTAGACAGACCATATCCAATTCAGGTCC
TCTTTCTGAGAATGTACTGATGCATCACACAGTCACACCAGTTCCACAAGTATTTAAGGAGGAGATTTCTTATAA
GTTCTGACCAAACATAAAGAGCACTTCAAAAGTGACCATGGTCCAGCCATATGGGTTAAGCCAATATAGTGGA
(B)
Color code
Methylation %
0
10
20
30
40
50
60
70
80
90
100
Foxp3– control
Foxp3+ untreated
Foxp3+ extr p.o.
Foxp3+ VacA i.p.
Foxp3+ untreated
Foxp3+ inf
Foxp3+ untreated
Foxp3+ inf
TSDR-3
TSDR-4
TSDR-5
TSDR-6
TSDR-7
TSDR-8
TSDR-9
TSDR-10
TSDR-11
TSDR-12
Experiment 1
Experiment 2
Experiment 3
(C)
Foxp3+ T cells
CD4+ T cells
CD8+ T cells
% of TCRβ+CD4+
% of live CD45+
% of live CD45+
– VacA i.p. Extr p.o. Inf
(D)
Foxp3
IL-10
TGF-β
Gene expression relative to Hprt
– VacA i.p. Extr p.o. Inf

3.4 | *Helicobacter pylori* infection, as well as extract and VacA treatment induce demethylation of the Treg-specific demethylated region in FoxP3$^+$ Tregs, promoting their lineage commitment and suppressive activity

Tregs have been implicated in asthma protection in *H. pylori*-infected mice and are numerically and functionally different in naïve and infected animals.[18-20] To assess whether the interventions shown here to confer food allergy protection affect the differentiation and stability of Tregs, we set out to quantify the methylation status of CpG motifs (Figure 4A) localized within a region of the *FOXP3* locus termed TSDR because of its selective demethylation in lineage-committed (stable) Tregs.[25,26] Tregs from pooled mesenteric lymph nodes of 6-8 male mice per group were FACS-sorted based on their CD4 and FoxP3 expression, and subjected to genomic DNA extraction, bisulphite conversion and TSDR-specific pyrosequencing. CD4$^+$ FoxP3$^-$ T cells were sorted and analysed in parallel. FoxP3$^-$ T cells exhibited a more or less complete methylation of the TSDR, with on average 96 % methylated CpG motifs (Figure 4B and data not shown); their methylation did not change upon intervention (data not shown). In contrast, FoxP3$^+$ T cells exhibited strong differences in their methylation status: whereas FoxP3$^+$ T cells that had been harvested from naive mice showed methylation levels varying between 25 and 50 % depending on the experiment, this level decreased to 10 % upon extract treatment, 10-17 % upon live infection and down to 1 % upon VacA treatment (Figure 4B). As reported previously,[25,26] the CpG motifs within the TSDR showed very consistent methylation patterns within one sample, indicating an all-or-nothing mechanism of demethylation that encompasses the entire locus (not all regions could be analysed in all samples though, Figure 4B). Furthermore, flow cytometric analysis of MLN T cells revealed somewhat higher frequencies of FoxP3$^+$ cells among all CD4$^+$ T cells in VacA-treated and neonatally infected mice, whereas overall CD4$^+$ and CD8$^+$ frequencies were similar (Figure 4C). CD4$^+$CD25$^+$ Tregs sorted from the MLNs of the same mice receiving interventions expressed modestly more FoxP3 and IL-10 and/or TGF-β than Tregs from untreated controls, confirming that TSDR demethylation has functional consequences with respect to Treg expansion and regulatory cytokine expression. We conclude from these data that epigenetic marks leading to stable expression of FoxP3, and to the definitive lineage commitment of Tregs, reflect the suppressive effects of *H. pylori*-specific treatments on food allergy in the models described above.

4 | DISCUSSION

Although food allergy represents an important human disorder of increasing prevalence in most parts of the world and *H. pylori* is known to be protective against allergies with respiratory tract manifestations,[10,18-20,27,28] little experimental or epidemiological evidence is available that addresses a possible inverse correlation, or protective effect, of *H. pylori* in food allergy. One of the reasons for this lack of data probably lies in the fact that no universally accepted food allergy model exists that would faithfully reflect all or most aspects of the human condition. We have adapted and optimized four complementary food allergy models, using various routes of sensitization and challenge, two different common food allergens and two mouse strains, to investigate in a comprehensive manner whether the presence of *H. pylori* affects clinically relevant food allergy symptoms, in either a beneficial or detrimental way. We found no strong evidence for a promoting role of *H. pylori* in food allergy, as had been suggested in two observational studies in humans.[14,15] In contrast, live *H. pylori* infection or extract or VacA treatment conferred detectable protection against anaphylactic symptoms in several of the models; the effects on clinical symptoms were associated with protective effects on systemic parameters of food allergy, such as mast cell protease levels in serum and allergen-specific IgG1 levels in some but not all models. The effects of *H. pylori* on food allergy were not as impressive as its beneficial effects in models of allergic asthma in which neonatal exposure to the bacteria essentially reduces parameters of airway inflammation, airway hyperresponsiveness and goblet cell metaplasia to almost background levels.[18-20] The stronger effects in the asthma models may have to do with the so-called gut-lung axis, the idea of a special connection between the mucosal immune systems of the two organs that has been put forward to explain the effects of (deliberate or pathogenic alterations of) the gut microbiota on lung diseases.[29] We were able to link the protection against the airway hyper-reactivity, inflammation, eosinophilia and excessive mucus production that are hallmarks of allergic asthma to the strong suppressive activity of *H. pylori*-induced Tregs.[19,20] Here, we present a possible epigenetic correlate of the suppressive/protective activity of Tregs in allergy, as these cells—coming from an *H. pylori*-infected

FIGURE 4 Decreased methylation of the Treg-specific demethylated region (TSDR) and increase in regulatory cytokine expression upon *Helicobacter pylori*-specific treatment. (A) Schematic overview of the *FOXP3* locus with the TSDR upstream of the TSS (retrieved using BLAST). The CG-containing region is marked in blue and CG motifs covered by pyrosequencing are marked in bold red. (B) Methylation pattern of 10 CG dinucleotides within the TSDR, of FACS-sorted MLN-derived CD4$^+$Foxp3$^+$ and CD4$^+$FoxP3$^-$ T cells. Animals were treated as indicated. The methylation status of the individual CGs is colour-coded (left panel). White cells indicate sequences that failed to yield interpretable results due to technical problems. (C) Frequencies of FoxP3$^+$ Tregs among all TCRβ$^+$ CD4$^+$ T cells, and of CD4$^+$ and CD8$^+$ T cells among all CD45$^+$ leucocytes in the MLNs of mice treated from 7 d of age onwards with either VacA, or *H. pylori* extract, or live bacteria. Horizontal lines represent medians. (D) Expression of FoxP3, IL-10 and TGF-β by sorted CD4$^+$CD25$^+$ Tregs, as assessed by qRT-PCR and normalized to Hprt, of the mice shown in C. Means +SEM are shown

host—exhibit an epigenetic signature at their *FOXP3* locus that is consistent with stable expression of FoxP3 and stable lineage differentiation and functionality.[25,26] We have shown in earlier work that the induction of Tregs depends on the *H. pylori* virulence factor and immunomodulator VacA; VacA mutants fail to induce suppressive Tregs and to confer asthma protection, and VacA alone, administered in purified form, recapitulates many of the beneficial effects of live infection.[22,30] *H. pylori* VacA mutants fail to colonize persistently and at wild-type levels, and rather are cleared effectively by an overshooting T-helper-1 response. The immunomodulatory effects of VacA were confirmed in the food allergy models presented here and were reflected in the epigenetic signature of Tregs isolated from VacA-treated mice. The rationale for applying preventive treatments in contrast to therapeutic treatments (which would start after sensitization) with VacA was governed by our prior experience in asthma models, in which we reported preventive but not therapeutic efficacy of *H. pylori*-specific tolerization. In conclusion, *H. pylori* possesses strong immunomodulatory properties, mediated at least in part by the VacA protein, that allow it to modulate T cell responses directed at the infection itself, as well as at conspicuous food and environmental antigens. These properties are evident not only locally in the gastric mucosa, but systemically, and strongly affect the individual carrier's allergy risk.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

AK and AM contributed to study concept and design. AK conducted all animal experiments with help from SU and AD. AK, SF and JH performed analyses and interpretation of data. TLC provided critical materials. All authors critically revised the manuscript for accuracy. AK and AM wrote the study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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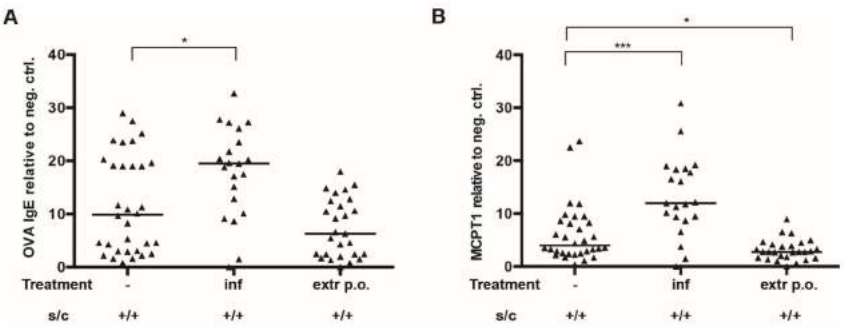
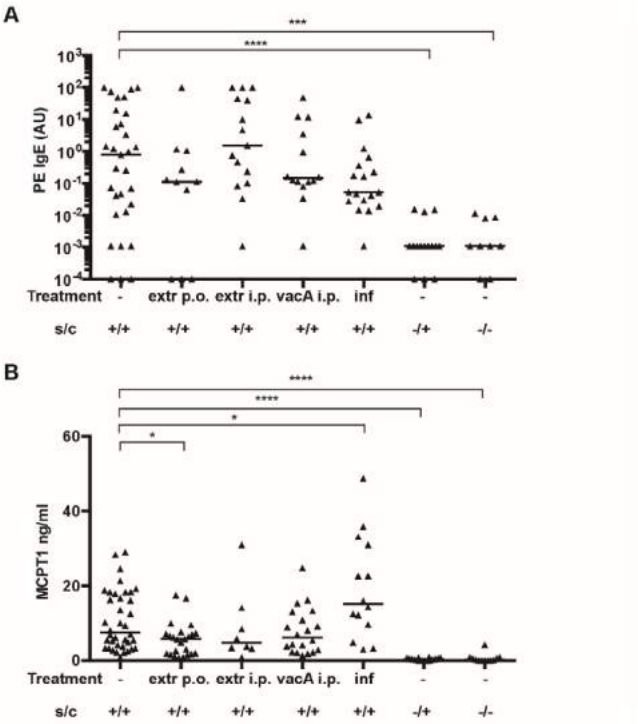
Supplemental Figures

Suppl. Figure 1



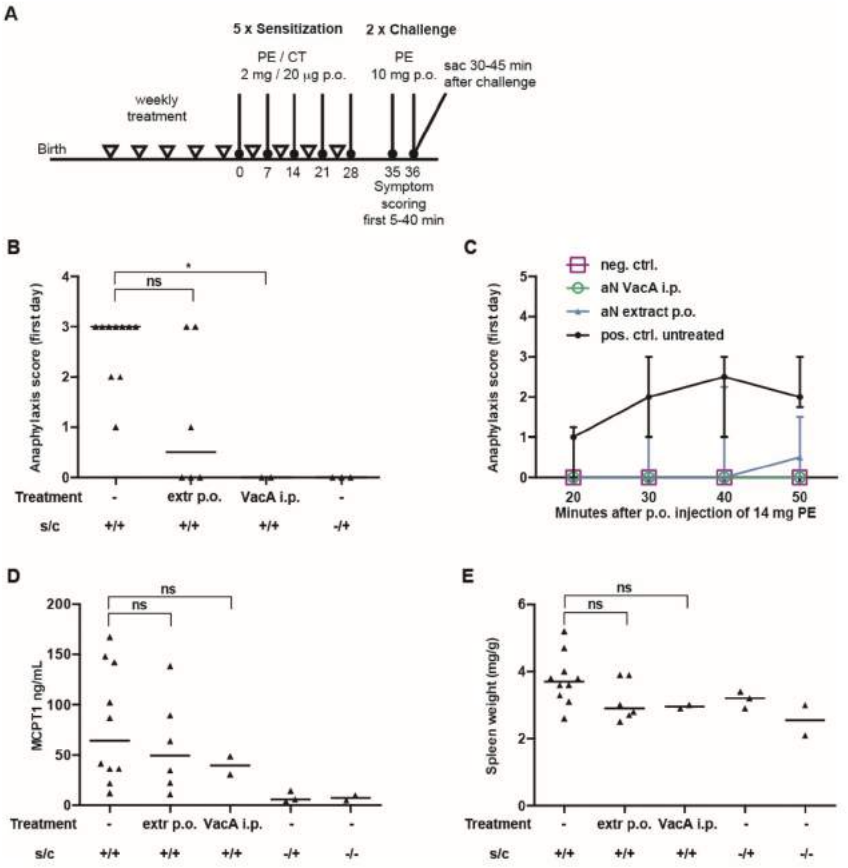
Suppl. Figure 1. OVA-specific IgE and mast cell protease (MCPT) 1 levels are reduced upon *H. pylori* extract treatment in an OVA-induced food allergy model. Mice were sensitized, challenged and treated as described in Figure 1. (A) OVA-specific IgE levels in serum, as determined by ELISA, of animals that were sensitized and challenged (s/c) with OVA and infected or not with *H. pylori*. Additional mice received regular doses of *H. pylori* extract by oral gavage (p.o.). All values were normalized to the average negative control (challenged, but not sensitized mice) IgE level. (B) Mast cell protease 1 (MCPT1) concentration in serum, as measured by ELISA, of the mice shown in A, normalized to the average negative control value. In A and B, each symbol represents one mouse. Graphs show pooled data from 5 (in A) and 4 (in B) experiments. Horizontal lines indicate medians; the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout for the calculation of p-values. * $p<0.05$, *** $p<0.001$.

Suppl. Figure 2



Suppl. Figure 2. PE-specific IgE and mast cell protease 1 levels are partially reduced upon *H. pylori* extract treatment or infection. Mice were sensitized, challenged and treated as described in Figure 3. (A) PE-specific OVA-specific IgE levels in serum, as determined by ELISA, of animals that were sensitized and challenged (s/c) with PE and infected or not with *H. pylori*. Additional mice received regular doses of *H. pylori* extract by oral gavage (p.o.) or i.p., or were treated i.p. with VacA. (B) In A and B, each symbol represents one mouse. Graphs show pooled data from 5 (in A) and 6 (in B) experiments. Horizontal lines indicate medians; the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout for the calculation of p-values. * $p<0.05$, *** $p<0.001$.

Suppl. Figure 3



Suppl. Figure 3. PE-specific anaphylaxis and mast cell protease 1 levels are reduced upon *H. pylori* extract or VacA treatment in C3H mice. (A) C3H mice were sensitized, challenged and treated as shown in the schematic. Briefly, mice were sensitized orally once a week for five weeks with 2 mg PE adjuvanted with 20 µg cholera toxin followed by two oral challenges on two consecutive days with 14 mg PE. (B,C) Anaphylaxis symptoms were scored after the first

challenge. The highest score per mouse is shown in B, and the median scores are shown in C, along with interquartile range, of the indicated groups at 20, 30, 40 and 50 min post challenge. (D) Mast cell protease 1 (MCPT1) concentration in serum, as measured by ELISA, of the mice shown in B. (E) Spleen weights of the mice shown in B. In B, D and E, each symbol represents one mouse. Horizontal lines indicate medians; the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used throughout for the calculation of p-values. * $p<0.05$.

3.3 Trans-maternal exposure to *Helicobacter pylori* induces stable and highly suppressive regulatory T-cells and protects against allergic asthma

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Contribution: I contributed to the study concept and design, conducted all animal experiments, performed analyses and interpretation of data, and wrote parts of the manuscript.

Trans-maternal exposure to *Helicobacter pylori* induces stable and highly suppressive regulatory T-cells and protects against allergic asthma

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Abstract, 200 words; Text, 10,679 words.

Abstract

The trans-maternal exposure to tobacco, microbes, nutrients and other environmental factors shapes the fetal immune system through epigenetic processes. The gastric microbe *Helicobacter pylori* represents an ancestral constituent of the human microbiota that causes gastric disorders on the one hand, and is inversely associated with allergies and chronic inflammatory conditions on the other. Here, we investigate the consequences of trans-maternal exposure to *H. pylori*, *in utero* and/or during lactation, on the composition of the gastrointestinal microbiota, susceptibility to viral infection, predisposition to allergic asthma, and the development of immune cell populations in the lung and lymphoid organs. Perinatal exposure to *H. pylori* extract, or its immunomodulatory molecule VacA, confers robust protective effects against house dust mite-induced allergic asthma not only in the first, but also the second generation of offspring, but does not increase susceptibility to viral airway infection. Immune correlates of allergy protection include skewing of regulatory over effector T-cells, expansion of Treg subsets expressing CXCR3 or RORγt, and demethylation of the *FOXP3* locus. The composition and diversity of the gastrointestinal microbiota is measurably affected by perinatal *H. pylori* exposure. We conclude that exposure to *H. pylori* has consequences not only for the carrier, but also for subsequent generations.

Introduction

The prevalence of allergic asthma has increased dramatically in recent decades, with ~235 million people affected worldwide (1). Environmental and lifestyle factors that include diet, exposure to antibiotics, sanitary conditions, country of birth, exposure to pets and livestock, the delivery mode and breastfeeding, sometimes collectively referred to as the "exposome", have all been causally implicated in this trend (2, 3). Many of the environmental factors affecting asthma and allergy risk act early in life, i.e. leave their strongest marks on young adults, children, newborns and even the unborn fetus (2, 3). Others act not only on the exposed individual, but may manifest in subsequent generations. Examples of environmental factors that preferentially or exclusively act early in life include exposure to rural farming environments and livestock, which decreases the atopic sensitization and allergy risk of (directly exposed) children and the offspring of exposed pregnant mothers through mechanisms that appear to involve regulatory T-cells (4, 5). Similarly, exposure to dogs and cats was reported to be protective against allergic disease development in childhood due to a higher diversity of bacterial communities found in such households (6, 7). Other factors contributing to allergy risk early in life are the mode of birth (i.e. vaginal or Caesarean section delivery) as well as breast-feeding, which affect the establishment of a diverse human gut microbiota; recent studies suggest that microbial colonization of the GI tract and other mucosal surfaces may be initiated already *in utero*, continues at birth with the acquisition of microbes during vaginal delivery, and is completed during early postnatal life (8-11). In contrast, delivery by cesarean section results in a neonatal microbiota resembling the one

found on human skin, with delayed acquisition of the more complex typical microbiota of a healthy GI tract (12, 13). Cesarean section delivery has been associated with respiratory distress, asthma and atopy of the affected children (14, 15).

It is now well-established that allergic infants and children exhibit a reduced diversity of their gastrointestinal microbiota that is characterized by a predominance of *Firmicutes*, and members of the *Bacteroidaceae* family and specifically by increased numbers of *Bacteroides fragilis*, *Escherichia coli*, *Clostridium difficile*, *Bifidobacterium catenulatum*, and *Bifidobacterium longum*, and a lower prevalence of *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, and *Lactobacillus* species (2). Other studies have reported a lower prevalence of *E. coli* (16) and of *C. difficile* (17) in allergic individuals. In both developed and developing countries, the decreasing prevalence of immunomodulatory microbes such as intestinal helminths (18, 19) or of *Helicobacter pylori* (20-23) is significantly associated with an increased risk for allergic diseases, especially in pediatric populations. We have reported that neonatal infection of mice with *H. pylori* protects effectively against the airway hyper-responsiveness, pulmonary inflammation and goblet cell metaplasia that are hallmarks of experimental models of asthma induced by ovalbumin or house dust mite allergen sensitization and challenge (24-27). The experimental infection of adult animals had no or much weaker effects on allergy parameters, a finding that is in agreement with epidemiological data suggesting that older adults benefit less from harboring *H. pylori* than children, adolescents and young adults, and that the early-onset forms of asthma show a stronger inverse association with *H. pylori* than asthma manifestations later in life (20, 21, 28, 29). The underlying protective

mechanism involves tolerogenic subsets of dendritic cells that -in the context of an *H. pylori* infection- drive the differentiation and suppressive activity of regulatory T-cells (24, 25, 27, 30). Interestingly, the immunomodulatory activity of *H. pylori* depends on two determinants, the vacuolating cytotoxin VacA and γ-glutamyl-transpeptidase GGT, that are on the one hand required for the protective effects of the live infection, and on the other hand recapitulate many of the benefits associated with live infection when administered in purified form (26, 30, 31).

Here, we investigated whether perinatal exposure (via the mother) to immunomodulatory molecules of *H. pylori* that occurs *in utero* or during lactation recapitulates the benefits of direct *H. pylori* exposure early in life; to this end, we perinatally exposed animals to *H. pylori* and evaluated their response to allergens in models of allergic asthma, and determined the immune, microbial metagenomic and epigenetic consequences of perinatal exposure to *H. pylori* later in life.

Results

Perinatal exposure to *Helicobacter pylori* extract protects against house dust mite-induced allergic asthma

To address whether administration of *H. pylori* extract to pregnant or lactating mice protects their offspring against allergic asthma later in life, we intragastrically treated females with twice-weekly doses of *H. pylori* extract generated by non-denaturing pressure homogenization. *H. pylori* extract was orally administered either during the three weeks of pregnancy, or during lactation, or both, with the first two treatments requiring litter swapping at birth (see schematic in suppl. Figure 1A). All offspring received an intranasal dose of house dust mite (HDM) allergen for the purpose of allergic sensitization at six weeks of age, followed on days 8-12 post-sensitization by five consecutive intranasal HDM challenge doses (suppl. Figure 1B) and, at day 15, by the endpoint assessment of bronchoalveolar leukocyte infiltration and eosinophilia, pulmonary inflammation and goblet cell metaplasia. Both pre- and post-natal exposure of the offspring (i.e. *in utero* or during lactation), as well as the combined treatment, efficiently protected the offspring against all assessed parameters of allergic asthma, with strongly reduced overall leukocyte infiltration of the bronchoalveolar lavage fluid (BALF), strongly reduced eosinophilia, lower inflammation scores and fewer PAS-positive goblet cells counted in the airway epithelium (Figure 1A-F). HDM-specific serum IgE titers and the pulmonary expression of the Th2 cytokine IL-13 were reduced as well (suppl. Figure 1C). The parallel administration of *E. coli* extract had no measurable effect on the parameters of allergic asthma, suggesting that the protection is specific to *H. pylori* (Figure 1A-F). Interestingly, we found the protective effects of extract exposure *in utero* or during

lactation to be quite comparable in magnitude to the direct intraperitoneal or intragastric treatment of newborn pups with extract (suppl. Figure 1D-H).

To address whether perinatal extract treatment induces general immunosuppression and thereby increases the susceptibility of the offspring to pulmonary challenge with influenza A virus (IAV) (32), we infected perinatally (pre- and postnatally) treated offspring with 200 PFU (0.4 HAU) of IAV. The infection triggered clearly detectable weight loss as well as high titers of PR8-specific IgG, with high titers correlating well with more pronounced weight loss (suppl. Figure 1I-K). The re-stimulation of lung leukocyte preparations with the influenza antigens NP1 and HA revealed strong influenza-specific $CD8^+$ T cell responses (suppl. Figure 1L). Perinatal *H. pylori* extract treatment did not measurably affect the magnitude of the IAV-specific readouts (suppl. Figure 1I-L), suggesting that the reduced severity of allergic asthma is not likely to be due to general immunosuppression.

Postnatal exposure to the *H. pylori* immunomodulator VacA protects against house dust mite-induced allergic asthma

We have previously attributed the allergy-protective effects of *H. pylori* to its secreted immunomodulator VacA, which is both necessary and sufficient for conferring protection against ovalbumin and house dust mite-induced allergic asthma on the one hand, and food allergy driven by ovalbumin or peanut allergen exposure on the other (26, 30, 33). As intraperitoneal injection was not feasible in pregnant dams, we administered VacA purified from culture supernatants of *H. pylori* either intraperitoneally during lactation only (Figure 2) or orally during pregnancy and lactation (suppl. Figure 2). Both treatments, administered twice

weekly, efficiently reduced the bronchoalveolar infiltration and eosinophilia, as well as lung inflammation and goblet cell metaplasia associated with HDM-induced allergic asthma (Figure 2A-E; suppl. Figure 2A-E). As observed with *H. pylori* extract, maternally administered VacA was as beneficial in terms of reducing allergy severity as direct intraperitoneal treatment of the offspring with VacA (suppl. Figure 1D-H). The combined results suggest that the *H. pylori* immunomodulator VacA has potent activity in suppressing hallmarks of allergic asthma not only when present in the context of a live infection, but also when administered in purified form to pregnant or lactating dams, or to pups during the first weeks of life.

To address whether exposure of the offspring to (however minuscule) amounts of extract *in utero* or during lactation were required for the protective effects, or tolerizing treatment of the mother from her own neonatal period onwards was sufficient to confer protection, we initiated the treatment of prospective mothers at 6 days of age, but stopped it before mating. The unexposed offspring of such tolerized mothers did not show evidence of protection against HDM-induced asthma (suppl. Figure 2F-J), indicating that exposure to extract via the placenta or the milk is a prerequisite for protection.

Perinatal exposure to *H. pylori* extract skews lung T-cell responses towards regulatory T-cells

We next performed a detailed analysis of the steady state lung lamina propria T-cell and myeloid compartments by multicolor flow cytometry to identify immune correlates of the differential propensity of extract-treated and control mice to respond to HDM allergen. Mice that had been exposed perinatally (i.e. *in utero* and

until weaning) to *H. pylori* extract exhibited generally lower pulmonary $CD4^+$ T-cell frequencies and lower Th1 and Th17 frequencies -as assessed by intracellular cytokine staining for the signature cytokines IFN-γ and IL-17 of *ex vivo* re-stimulated leukocyte preparations- than did the PBS-exposed offspring (Figure 3A,B). Although the overall $Foxp3^+$ Treg frequencies did not differ among the two treatment groups, extract-exposed mice exhibited significantly higher frequencies of pulmonary neuropilin-negative Tregs (which are enriched for peripherally induced iTregs) and of two Treg subsets that are associated with particularly high suppressive activity, i.e. $CXCR3^+$ and $ROR\gamma t^+$ Tregs (Figure 3C-F) (34-37). In contrast, the very abundant $IRF4^+$ Treg subset was not significantly different, and if anything, underrepresented, in the lung lamina propria of extract-exposed mice (Figure 3G). The shifts in T-cell populations appeared to be specific to the lung and were not observed in the mesenteric lymph nodes (MLNs; suppl. Figure 3A-G).

We next asked whether DC subsets were quantitatively or functionally different in the lung lamina propria of extract- and PBS-exposed mice. DCs are critically required for the development of peripheral tolerance to antigens and allergens, and we have reported previously that a $CD103^+$ subset of DCs is over-represented in the lungs of mice that are protected against allergic asthma due to live infection with *H. pylori* (30). Although the overall frequencies of $MHCII^+$ $CD11c^+$ pulmonary lamina propria DCs were somewhat lower in extract-treated relative to control animals (Figure 3H), the ratio of $CD103^+$ to $CD11b^+$ DCs, which represent the two major resident DC populations, was higher (Figure 3I). As observed for T-cells, DC populations in the MLNs did not differ measurably depending on the perinatal exposure (suppl. Figure 3H-K). To assess the functionality of DCs in the lung, we administered recombinant

ovalbumin (OVA) protein that is coupled either to a constitutive fluorophore (AF647) or to a fluorophore that requires OVA processing and presentation in the context of MHCII molecules (DQ-OVA). Perinatal extract exposure did not measurably impair the ability of $MHCII^{+}CD11c^{+}$ pulmonary lamina propria DCs to sample or to process OVA (suppl. Figure 3L,M); however, the OVA-positive or DQ-OVA-positive DC populations recapitulated the general trend toward over-representation of $CD103^{+}$ DCs (suppl. Figure 3N-Q). Overall, our immunological profiling efforts reveal the skewing of T-cell responses towards regulatory T-cells in extract-treated animals, which is consistent with the observed shifts favoring tolerogenic over immunogenic DC subsets.

Perinatal exposure to *H. pylori* extract or VacA affects the diversity and composition of gastrointestinal bacterial community structures

To address whether the perinatal exposure to *H. pylori* extract or VacA not only has consequences for allergy severity and immunological correlates of protection, but may also affect the gastrointestinal microbiota, we performed 16S rRNA sequencing of 194 samples from the stomach, ileum, cecum and colon of 50 mice that were subjected to perinatal extract, VacA, or PBS treatment (i.e. their mothers were orally gavaged with extract, PBS or VacA throughout pregnancy and lactation). We obtained an average of 6407 reads per sample. The sequencing depth was comparable across organs and to include 190 (i.e. 98% of) samples, we rarified to 2,000 reads, but results were similar with greater read depth and fewer samples (data not shown). In total, we identified 512 observed operational taxonomic units (OTUs). Examination of beta-diversity using unweighted UniFrac analysis revealed a

clear segregation of samples driven by anatomical site as well as by treatment modality (suppl. Figure 4A,B). For each organ studied, samples clustered significantly by the treatment group; this was most pronounced in the stomach and ileum (Figure 4A,C). Linear discriminant analysis (LDA) effect size (LEfSE) confirmed significant differences in relative abundances related to treatment. The largest differences were observed in the stomach and ileum; both VacA and extract treatment favored depletion of Firmicutes and Bacteroidetes in the stomach (Figure 4B), while several taxa were strongly over-represented at the class and order level (Figure 4B,D). In the stomach, VacA treatment resulted in depletion of Allobaculum and enrichment of Ruminococcaceae Clostridium (Figure 4B). VacA treatment was associated with depletion of the taxa Clostridiaceae Candidatus Arthromitus (Segmented Filamentous Bacteria) and treatment with either extract or VacA resulted in a depletion of Akkermansia and Desulfovibrio species in the ileum (Figure 4D). The results from our metagenomics analysis thus suggest that perinatal exposure to *H. pylori* immunomodulators has clearly discernible effects on bacterial communities in the gastrointestinal tract much later in life, which may either be a cause or consequence of the skewing of steady state immune parameters towards regulatory branches of the immune system. To assess possible causality, we transplanted the cecal content of perinatally extract-exposed adult animals into neonates and subjected these animals to HDM sensitization and challenge as adults. Cecal transplantation was not sufficient to confer protection against allergic asthma (suppl. Figure 4C-G), suggesting that the changes in the cecal tract microbiota are probably a consequence rather than the cause of the immunomodulation that manifests in reduced allergy symptoms.

The systemic depletion of regulatory T-cells reduces the protective effects of *H. pylori* extract on house mite-induced allergic asthma

Having observed that, in the steady state, perinatally extract-treated mice exhibit significantly higher frequencies of pulmonary Treg subsets with known or presumed suppressive activity than untreated controls, we next set out to deplete Tregs systemically during the challenge phase of our protocol of allergic asthma. To this end, HDM-sensitized mice expressing GFP and the diphtheria toxin (DT) receptor under the control of the *Foxp3* locus (FoxP3^{DTR})(38) received a total of four doses of DT a few days prior to, and during, allergen challenge. The efficiency of Treg depletion, as judged by flow cytometric analysis of the residual GFP^{+}CD4^{+} T-cell populations, was >80% in the lung and MLNs even when assessed three days after application of the last DT dose (suppl. Figure 5A,B). The beneficial effects of perinatal exposure to *H. pylori* extract on allergen-induced bronchoalveolar infiltration and eosinophilia, and on pulmonary inflammation and goblet cell metaplasia, were largely abolished by Treg depletion (Figure 5A-E), as were the effects on HDM-specific serum IgE titers and the pulmonary expression of the Th2 cytokine IL-13 (suppl. Figure 5C,D). However, interpretation of the results is complicated by the fact that, also in mock-treated FoxP3^{DTR} mice of the positive control group, the depletion of Tregs aggravated several readouts of allergic asthma (Figure 5A-E). While demonstrating that Tregs are required for the suppressive effects of perinatal tolerization with *H. pylori* extract, the results thus also show that normal complements of Tregs have an important role in suppressing excessive allergen-specific immune responses.

Perinatal extract and VacA treatment results in an enhanced demethylation of the TSDR in Foxp3$^+$ Tregs

Tregs contribute to asthma protection in mice that are perinatally tolerized with *H. pylori* extract or with VacA (Figure 5), and the frequencies of specific Treg subsets are elevated in the lungs as a consequence of the treatment (Figure 3). We therefore set out to assess whether the same interventions affect the epigenetic processes driving the differentiation and stability of Tregs. To this end, we quantified the methylation status of CpG motifs (Figure 6A) localized within an intronic enhancer region of the *Foxp3* locus termed TSDR because of its selective demethylation in lineage-committed (stable) Tregs (39, 40). Tregs from pooled MLNs of six to ten male adult mice per group were FACS-sorted based on their CD4 and Foxp3 expression, and subjected to genomic DNA extraction, bisulfite conversion and TSDR-specific pyrosequencing. CD4$^+$Foxp3$^-$ T-cells were sorted and analyzed in parallel. We used MLN Tregs since the cell numbers that were obtained from the lungs were far too low for methylation analyses. As expected, Foxp3$^-$ T-cells exhibited a demethylated TSDR, with ~95% methylated CpG motifs (Figure 6B); their methylation did not change upon intervention (data not shown). In contrast, Foxp3$^+$ T-cells exhibited strong differences in their methylation status: whereas Foxp3$^+$ T-cells that had been harvested from naive mice showed methylation levels varying between 25-40% depending on the experiment, this level decreased to 5-15% due to perinatal intervention with extract or with VacA (Figure 6B). The ten analyzed CpG motifs of the TSDR showed very consistent methylation patterns within one sample, indicating an all-or-nothing mechanism of demethylation that encompasses the entire locus (note that not all regions could be analyzed in all samples, Figure 6B). Furthermore, a

qRT-PCR-based analysis of the Treg-specific transcripts FoxP3, IL-10 and TGF-β conducted on $CD4^+Foxp3^+$ Tregs, sorted in parallel from lungs and MLNs, showed modestly higher TGF-β, but not Foxp3 and IL-10 expression due to the two interventions (suppl. Figure 6A,B). We conclude from these data that epigenetic marks leading to stable expression of Foxp3, and to the definitive lineage commitment of Tregs, reflect the suppressive effects of *H. pylori*-specific perinatal interventions on allergic asthma.

The decreased susceptibility to allergic asthma by exposure to *H. pylori* extract is inter-generationally transmitted to the F2 generation

The enhanced demethylation of the TSDR in $Foxp3^+$ Tregs from extract- and VacA-treated mice encouraged us to set up additional crosses, this time between perinatally extract-exposed (i.e. through oral gavage of their mothers) and naive offspring. Strikingly, we found that the severity of HDM-induced allergic asthma was reduced even in the F2 generation born to the perinatally extract-exposed offspring, i.e. to the grandchildren of extract-treated dams (Figure 7A-E). It did not matter in this context whether only the father, or only the mother, or both parents of HDM-sensitized and challenged offspring had been exposed *in utero* and during lactation to the *H. pylori* extract administered to their mother (Figure 7A-E). These experiments do not rule out that the F2 offspring benefited in terms of their reduced allergy severity because the gametes that form in the developing fetus *in utero* had been exposed to the tolerance-promoting components of *H. pylori* extract. However, the results provide experimental evidence for the observation that allergy risk in

humans is strongly affected by environmental exposures even across more than one generation (41).

Discussion

In this study, we present the first experimental evidence for an intergenerational effect of the perinatal exposure to *H. pylori*-derived immunomodulatory molecules during pregnancy and lactation on several parameters of atopic sensitization and allergen-induced asthma. We further report several immunological, meta-genomic and epigenetic correlates of perinatal *H. pylori* exposure that may at least partly account for the observed protective effects. The acquisition of *H. pylori* in early life has been implicated in epidemiological as well as experimental studies in having a clearly detectable influence on allergy risk and severity, especially in children and young adults and their murine counterparts in experimental models of allergic asthma (20-27). Here, we have extended these findings and focused on possible exposures occurring even earlier in life, i.e. *in utero* and during lactation. To exclude the risk of vertical transmission of the infection from mother to offspring, a route that has been identified as the main route of *H. pylori* transmission in humans (42), we instead opted to treat pregnant or lactating dams with *H. pylori* extract or purified VacA, the main immunomodulatory molecule secreted by *H. pylori* to skew T-cell responses towards Tregs and promote persistent colonization (26).

The concept that direct perinatal exposure to specific intact microbes may be beneficial with respect to allergic asthma risk and severity is not new, and has been tested extensively in experimental models with bacteria isolated from the farming environment, such as *Acinetobacter lwoffii*, *Lactobacillus lactis*, *Bacillus licheniformis*, and *Staphylococcus sciuri* (3, 43-45). Trans-maternal asthma-protective effects as shown here for *H. pylori* (with exposure occurring exclusively via the mother) were reported in experimental models with *Acinetobacter lwoffii*, and in

those models were dependent on intact TLR signaling and IL-6, TNF-α, and IL-12 production in the mother and epigenetically regulated IFN-γ production in the offspring (46, 47). Observational studies in human subjects lend further support to the idea that prenatal microbial exposures (e.g. to farming environments) have extensive effects on innate and adaptive immune function that are regulated at least in part through epigenetic mechanisms (48, 49). Regulatory T-cells have been implicated in some of the benefits associated with prenatal exposure (via the mother) to farming environments; their numbers and functionality in cord blood were found to be significantly higher in offspring of farm-exposed relative to control mothers, which in turn was associated with lower Th2 cytokine secretion and lymphoproliferation upon innate stimulation (48). Conversely, reduced Foxp3 expression in fetus-derived placental tissues appears to predict allergic disease in infancy (49).

Three pieces of evidence implicate Foxp3$^+$ Tregs in the beneficial effects of perinatal trans-maternal exposure to *H. pylori* and its immunomodulator VacA. On the one hand, we observe in the steady state (i.e. prior to allergen sensitization and challenge) that the frequencies of specific Treg subsets -all associated with particularly strong suppressive activities (34-37)- in the lung are higher than without such perinatal exposure. Secondly, we detected enhanced demethylation of the TSDR within the *Foxp3* locus, which serves as an indicator of stable, irreversible differentiation towards the Treg lineage (39, 40), only in Tregs from *H. pylori*-exposed, not from naïve mice. Finally, the specific and selective depletion of Foxp3$^+$ Tregs by DT administration during the challenge phase of the asthma protocol abrogates the benefits of trans-maternal exposure, indicating that the suppression

of allergen-specific Th2 responses (and their signature cytokines, as determined in this study for IL-13) is driven by Tregs. The higher infiltration of *H. pylori*-exposed lungs with certain subsets of Tregs in the steady state correlates well with their lower T-effector cell frequencies; the preferential priming (or local expansion) of Tregs over effector T-cells may be attributable to the skewed ratio of DCs with tolerogenic vs. inflammatory activities. The $CD103^+$ DCs that are over-represented in the lungs of *H. pylori*-exposed mice relative to $CD11b^+$ DCs depend on the transcription factor basic leucine zipper ATF-like 3 (BATF3), and are known for their potency in driving $CD8^+$ T cell immunity (50-52) and for their role in priming Treg differentiation through the production of retinoic acid (53, 54). Although their exclusive, non-redundant role in promoting Treg-driven tolerance was recently questioned in an oral tolerization model (55), we have found their over-representation in the lung to be indicative of protection against asthma in experimental models (30). Furthermore, the deficiency of $CD103^+$ DCs in $BATF3^{-/-}$ mice abrogates the protective effects of live infection (30), and also of direct VacA treatment (unpublished data). More work will be required to elucidate which of the various differentially represented Treg subsets depend on $CD103^+$ DCs for their priming or local expansion, and whether BATF3 proficiency is required for protection of trans-maternally exposed offspring to allergic asthma.

We found three Treg subsets to be over-represented in the lungs of *H. pylori*-exposed animals despite similar overall $Foxp3^+$ Treg frequencies. These were $Nrp1^-$ Tregs, i.e. cells that presumably arose in the periphery in a thymus-independent manner, as well as $ROR\gamma t^+$ and $CXCR3^+$ subsets of both peripherally induced Tregs and, to a lesser extent, thymus-dependent natural Tregs. Notably, in contrast to the

GI tract (37), we found that RORγt and CXCR3 were not generally co-expressed in the pulmonary Treg compartment. Expression of the chemokine receptor CXCR3 is known to be driven by the transcription factor T-bet and can be used as a marker of cells that have at some stage of their ontogeny (transiently or stably) expressed T-bet (37). T-bet-dependent, CXCR3$^+$ Tregs have recently been shown to develop in parallel to T-bet-positive Th1 cells during infection with *Listeria monocytogenes* (37), which similar to *H. pylori* is a strong Th1 inducer. Furthermore, the loss of T-bet$^+$ Treg cells (by selective depletion of Foxp3 expression in T-bet$^+$ Tregs) is sufficient to induce systemic autoimmunity, notably with strong T-effector cell infiltration into the lung (37). Our immunophenotyping data, albeit largely descriptive, are consistent with the novel concept that Tregs co-expressing FoxP3 and T-bet/CXCR3 have an essential immunosuppressive function and further suggest that not only autoimmunity, but also Th2-driven allergy, is controlled by these cells. In contrast, we found IRF4$^+$ Tregs to be under- rather than over-represented in the lungs of *H. pylori*-exposed animals, largely as a consequence of the elevated frequencies of the other two (RORγt$^+$ and CXCR3$^+$) populations. As IRF4 expression in Tregs had previously been shown to endow these cells with the ability to selectively suppress Th2 responses (56), we had expected IRF4$^+$ Tregs to increase in the setting of perinatal *H. pylori* exposure; however, this was not the case.

Interestingly, the observed shifts in immune cell populations that are a hallmark of mice trans-maternally exposed to *H. pylori* did not affect antiviral responses, which were normal in a model of pulmonary influenza infection. We further speculated that the differences in CD4$^+$ T-cell populations, in particular those affecting T-effector to Treg ratios, would have an influence on microbial communities in the

upper airways as well as the GI tract. Indeed, we found both VacA and *H. pylori* extract exposure to measurably affect microbial communities in all examined sites of the GI tract. Although the described cecal transplantation experiments appear to rule out a causal role for the altered microbiota composition in preventing allergic asthma, we found interesting that some of the shifts occurred in taxa that have previously been associated with healthy vs. diseased states, such as the segmented filamentous bacteria.

The most striking observation made in the course of our studies related to the intergenerational transmission of asthma protection. Such effects had previously been reported mostly for exposure to tobacco smoke, where not only maternal but also grand-maternal smoking is associated with increased risk for childhood asthma in humans (41). The results of the observational studies in humans have been corroborated in a rat model of nicotine-induced asthma, in which a trans-generational transmission to the F2 and F3 generations could be demonstrated following perinatal nicotine exposure of F0 dams (57). Given the striking beneficial (and detrimental) effects of perinatal exposures on F1, and possibly even F2 and F3 generations, various attempts have been made to introduce and test interventions during pregnancy and lactation. In particular, numerous studies have examined the effects of vitamin D supplementation, with controversial success (summarized in (58)). A recent study of n-3 long-chain polyunsaturated fatty acid supplementation during the third trimester of pregnancy showed a reduction of the absolute risk of persistent wheeze or asthma and infections of the lower respiratory tract in offspring by one third (59). Vitamin C supplementation throughout pregnancy was shown to offset some of the negative effects of maternal smoking on the lung

functions and wheezing of the offspring (60), an effect that may be attributable to the reversal of smoking-induced DNA methylation changes as evidenced by bisulfite sequencing of differentially methylated loci in placenta, cord blood and buccal samples of offspring (61). Finally, supplementation with pro- and pre-biotics, especially transiently colonizing bifidobacteria and lactobacilli, during pregnancy has been tested extensively, with mixed success (summarized in (2)).

Collectively, our findings indicate that trans-maternal pre- and postnatal exposure to *H. pylori* and its main immunomodulator VacA has robust effects on the severity of allergic asthma much later in life, which at least partly can be explained by the suppressive activity of the pulmonary Tregs that are induced under such conditions. Among the useful indicators of the reduced asthma risk associated with perinatal *H. pylori* exposure are shifts in the microbiota composition of various sites of the GI tract, as well as the epigenetic signature of the *Foxp3* locus, which indicates qualitative or at least quantitative differences in the stability and functionality of Tregs due to this treatment. Importantly, perinatal exposure to *H. pylori* does not result in generalized immunosuppression and an elevated susceptibility to viral (pulmonary) infection; rather, acute infection with the lung pathogen influenza A virus readily breaks perinatally induced immune tolerance. We propose that the common human amphibiont *H. pylori* functions as an integral part of the early life "exposome" that skews the developing immune system towards immune tolerance.

Methods

Animal experimentation

C57BL/6 mice were purchased from Janvier and Foxp3$^{eGFP\text{-}DTR}$ (B6.129(Cg)-Foxp3tm3$^{(DTR/GFP)Ayr}$/J, 016958) mice were purchased from the Jackson Laboratory and included in asthma experiments at 5-8 weeks of age. For the induction of acute house dust mite (HDM)-induced asthma, mice were briefly anesthetized and subsequently subjected to six intranasal injections of house dust mite extract (Greer laboratories, XPB70D3A25 Dermatophagoides pteronyssinus) on day 0 (1 µg) and on days 8-12 (10-15 µg depending on extract lot). Mice were sacrificed on day 15. Transgenic Foxp3-eGFP-DTR mice were intraperitoneally treated 4 times with 1 µg diphteria toxin (DT, Sigma D0564-1MG) during days 5-12 of the HDM-asthma protocol. At the study endpoint, blood was collected and serum prepared. Lungs were lavaged via the trachea with 1 mL of PBS. Broncho-alveolar lavage fluid (BALF) cells were counted using trypan blue dye exclusion. Differential cell counts of macrophages, lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with the Microscopy Hemacolor-Set (Merck). One lung lobe was collected, homogenized and the total protein isolated, and the concentration determined using the BCA Protein Assay Kit (Thermo Scientific 23227). For histopathology, lungs were fixed by inflation and immersion in 10% (vol/vol) formalin and embedded in paraffin. Tissue sections were stained with H&E and periodic acid-Schiff and examined in blinded fashion on a BX40 Olympus microscope. Peribronchial inflammation was scored on a scale from 0 to 4. PAS-positive goblet cells were quantified per 1 mm of basement membrane.

For the production of *H. pylori* extract, bacterial cultures of the PMSS1 were pelleted, washed with PBS, and subjected to three freeze-thaw cycles and homogenization using a pressure cell homogenizer (Stansted SPCH-18). The same procedure was used to produce *E. coli* (BL21, DE3) extract. The homogenate was centrifuged at 3000 x g, the resulting supernatant was filter-sterilized, and the protein concentration was determined as above. Oligomeric s1m1 type VacA was purified from culture supernatants of *H. pylori* strain 60190 expressing Strep-tagged VacA via a streptactin resin as described previously (62). The dosage of extract and VacA was adjusted to the age of the mice and the application mode: extract p.o. 50-200 µg, VacA i.p. 5-20 µg, VacA p.o. 10-20 µg. Neonates were treated with VacA or extract 1-2 times per week. Dams were treated 2-3 times per week during the entire pregnancy and lactation phase. For microbiota transplantation, the cecal contents of trans-maternally treated or untreated adult mice was isolated, suspended in PBS, the weight per ml PBS adjusted, centrifuged at 300 x g and filtered through a cell strainer before gavaging it to 7 day-old pups.

IL-13 and HDM-specific IgE ELISA

The concentration of IL-13 in lung homogenate was determined by Mouse IL-13 ELISA kit (eBioscience 88-7137-88). Measurement of serum HDM-specific IgE was achieved by coating high affinity 96-well plates with 100 µl 25 µg/ml HDM extract in carbonate-bicarbonate coating buffer overnight at 4°C. After washing and blocking, diluted samples were incubated for 2 hours, before washing again and adding an HRP-coupled IgE antibody (GeneTex GTX77227) for detection. Wells were washed again and HRP substrate was added and the absorbance measured on a plate reader.

Arbitrary units were calculated by using a standard curve determined by a serial dilution of a serum mix processed on the same plate.

TSDR methylation analysis

Total mesenteric lymph node cells of male C57BL/6 mice were isolated by means of collagenase type IV (Sigma C5138) digestion and filtering through a cell strainer. After fixation, permeabilization and washing, the cells were stained with anti-mouse CD4-FITC (Biolegend 100510) and Foxp3-APC antibodies (eBioscience 17-5773-82). $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells were sorted on a FACS Aria. Genomic DNA was isolated from sorted cell subsets using the NucleoSpin® Tissue kit (Macherey-Nagel). An additional step was added to the manufacturer's protocol to remove formaldehyde-induced crosslinking. Briefly, Chelex-100 beads (Biorad) were added after the lysis step and incubated at 95°C for 15 min in a shaker. Chelex-100 beads were spun down and the supernatant was transferred to a fresh tube. After addition of an adjusted amount of 100 % ethanol the following purification steps were performed according to the manufacturer's protocol. Genomic DNA was converted with bisulfite using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. The Treg-specific demethylated region (TSDR) was amplified by PCR and analyzed by pyrosequencing on a PSQ96MA (Qiagen) as described (63); primers for sequencing were (in 5' to 3' direction), S1: CCATACAAAACCCAAATTC, S2: ACCCAAATAAAATAATATAAATACT, S3: ATCTACCCCACAAATTT, S4: AACCAAATTTTTCTACCATT), which cover CpG motifs 3-12 of the TSDR core region.

Flow cytometric analysis and cell sorting for qRT-PCR

Total mesenteric lymph node cells of male C57BL/6 or Foxp3-eGFP-DTR mice were isolated by filtering through a cell strainer. Cells from perfused lungs were isolated by mincing the tissue followed by a digestion with collagenase type IA (Sigma, C9891-500MG) and mechanical disruption using a syringe. As a final isolation step, the lung suspension was applied to a cell strainer. To assess the DC allergen uptake and processing capacity, mice were intranasally challenged with 50 µl DQ-OVA (800 µg/ml, Thermo Fisher Scientific D12053) and AF647-OVA (800 µg/ml, Thermo Fisher Scientific 034784) ~ 15 hours before euthanization. After washing, the cells were stained in various combinations with anti-mouse CD4 PerCP/Cy5.5 or BV 785 (Biolegend 116012 or 100552), anti-mouse CD45 BV650 (Biolegend 103151), the fixable viability dye eFluor780 (eBioscience 65-0865-14), anti-mouse TCR β chain PE/Cy7 (Biolegend 109222), anti-mouse siglecF PE or BB515 or BV421 (BD 552126, 56514, 562681), anti-mouse CD16/CD32 Fc Block (Biolegend, 101302), anti-mouse CD11c BV605 (Biolegend 117333), anti-mouse CD11b PerCP/Cy5.5 (Biolegend 101228), anti-mouse CD103 PE (Biolegend 121406), anti-mouse I-A/I-E AF700 (Biolegend 107622), anti-mouse F4/80 APC (Biolegend 123116), anti-mouse CD8α BV510 or PE/Cy7 (Biolegend 100752 or 100722), anti-mouse Neuropilin-1 BV421 (Biolegend 145209), anti-mouse CXCR-3 BV510 (Biolegend 126527), anti-mouse CD3 Biotin (Biolegend 100244) in combination with Streptavidin BV711 (Biolegend 405241). After fixation, permeabilization and washing, cells were stained with anti-mouse Foxp3 BV421 (Biolegend 126419) or anti-mouse Foxp3 FITC (eBioscience 11-5773-82), anti-mouse RORγt PE-eFluor 610 (eBioscience 61-6981), anti-mouse IRF4 PerCP-eFluor 710 (eBioscience 46-9858-80). In some experiments cells were re-

stimulated *in vitro* in IMDM medium supplemented with Golgi-stop (BD 51-2092KZ), BFA (eBioscience 00-4506-51), PMA (Sigma P-8139) and ionomycin (Sigma I0634-1MG) before fixation and permeabilization for intracellular cytokine staining with anti-mouse IL-17 APC (Biolegend 506916) and anti-mouse IFNγ AF488 (Biolegend 505815). Samples were analyzed on a LSR II Fortessa instrument followed by detailed analysis using FlowJo software. For FACS-sorting of regulatory T-cells, mesenteric lymph node cells or lung cells from Foxp3$^{eGFP\text{-}DTR}$ mice were stained with the fixable viability dye eFluor780 (eBioscience 65-0865-14), anti-mouse CD4 BV711 (Biolegend 100550), anti-mouse SiglecF BV421 (BD 562681) and sorted for live CD4^{+}GFP^{+} cells on a FACS Aria. RNA of sorted cells was isolated using the RNeasy Mini Kit (Qiagen 74106), converted into cDNA, and subjected to TaqMan Real-Time PCR assay using the primers Mm03024075 (Hprt), Mm00475162 (Foxp3), Mm01178820 (Tgfb1) and Mm01288386 (IL10; all from Thermo Fisher Scientific). Samples were run on a Light Cycler 480 and normalized to the house-keeping gene *Hprt*.

Influenza A Infection

Seven-week old mice were anesthetized by intraperitoneal (i.p.) injection of 0.05 mg/kg Fentanyl (Sintetica), 5 mg/kg Midazolam (Dormicum, Roche), 0.5 mg/kg Medetomidin (Dorbene, Gräub) and infected intranasally with 200pfu of Influenza A H1N1 strain A/Puerto Rico/8/1934 (PR8, Charles River) diluted in 30µl PBS (Gibco). Anesthesia was antagonised by i.p. injection of 2.5 mg/kg Atipamezolin (Alzane, Gräub), 1.2 mg/kg Naloxon (Swissmedic), 0.5 mg/kg Flumazenil (Anexate, Roche). Mice were sacrificed by CO_2 asphyxiation on day 9 post-infection. Blood was

obtained by heart puncture. Lungs were perfused with cold PBS and extracted. Extracted lungs were cut into small pieces. After digestion with 2mg/ml Collagenase A (Sigma C9891) and 40µg/ml DNAse A (Roche 7002221) for 45min at 37°C, a single cell suspension was obtained by passing the tissue through a cell strainer of 40µm. Leukocytes were isolated by Percoll gradient centrifugation (GE Healthcare). Isolated leukocytes were restimulated in vitro in RPMI-1640 medium (Gibco) with 2µg/ml rat anti-mouse CD28 (BD 553294) and 10µg/ml PR8-specific epitopes $NP_{1366–374}$ and $HA_{211-225}$ or irrelevant control peptide $OVA_{257-264}$ (peptides&elephants). Brefeldin A (BD) was added after one hour of restimulation at 5µg/ml. After an additional incubation time of 4h, cells were stained with FITC-labelled rat anti-mouse CD8 (eBioscience, clone 53-6.7), fixed and permeabilized following manufacturer's instructions (BD Cytofix/Cytoperm kit, 554714) and intracellularly stained with PE-labelled rat anti-mouse IFNgamma (BD 554412). Cells were subjected to flow cytometric analysis using a FACS Canto II (BD). For the determination of PR8-specific IgG titres, adsorbent 96-well ELISA plates (NUNC) were coated with PR8 virus at $5x10^5$ pfu/ml overnight at 4°C. Virus was inactivated by UV irradiation of 2x 240 mJ. Blood was collected in Microtainer tubes (BD 365967) and spun at 3500 rpm for 10 min to obtain serum. Serial dilutions of serum in PBS were distributed in the ELISA plates. Bound IgG was detected with HRP-coupled goat anti-mouse IgG (Jackson Laboratories) and visualized by incubation with substrate (TMB, Sigma). The reaction was stopped with H_2SO_4 1N solution and plates were acquired on a Tecan infinite M200 Pro reader at 450 nm and 620 nm. Values at 620 nm were subtracted from readings at 450 nm.

Library Preparation

Gastric tissue and ileal, cecal, and colonic tissue and contents were collected after necropsy and immediately frozen in liquid nitrogen and stored at -80°C until DNA extraction. DNA was extracted using the DNeasy PowerSoil HTP 96 Kit (Qiagen), and the V4 region of the bacterial 16S rRNA gene amplified in triplicate using barcoded fusion primers (F515/R806)(64). Amplicon replicates were pooled, and the DNA was quantified using Quant-iT PicoGreen (Invitrogen). A maximum of 94 samples were then pooled at 20 nM concentration, purified using a Qiaquick PCR purification kit (Qiagen) and quantified using a Qubit 2.0 Fluorometer (Life Technologies). Finally, these samples were pooled at equal molar concentrations and sequenced on the Illumina MiSeq platform.

Microbiome Analysis

Utilizing QIIME 1.9.1, forward and reverse paired-end reads were trimmed and joined before being demultiplexed, filtered and analyzed. Open-reference OTU-picking was performed using the Greengenes Database Consortium (May 2017). Unweighted UniFrac distances were calculated and 2D-principal coordinate (PCoA) plots were generated in QIIME. Statistical significance was determined using the Adonis or Anosim tests. To determine OTUs that were significantly altered by treatment, a linear discriminate analysis (LDA) effect size (LEfSe) was performed. Taxa were classified as significantly different between groups when the log10 LDA score was >2.0, and the p-value <0.05 by the ANOVA test. Taxa were summarized at the genus level, and unclassified taxa were removed.

Statistical analysis

GraphPad Prism 6 was used for all statistical analyses. In almost all graphs symbols represent individual mice and horizontal lines indicate medians. The Mann-Whitney test was used throughout to assess for significant differences. Stars are used to indicate the level of significance according to the p-value: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Study approval

All animal experimentation was reviewed and approved by the Zurich Cantonal Veterinary Office (licenses 170/2014 to A.M. and 210/2014 to C.M.).

Author contributions

A.K. designed, performed and analyzed most of the experiments and co-wrote the manuscript; X.Z., A.A. and S.U. helped with animal experimentation. P.P. and C.M. performed viral infections. T.B. and M.J.B. generated and analyzed metagenomics data; C.T. contributed the lung histopathological analyses; T.L.C. provided critical materials. S.F. and J.H. performed TSDR bisulfite pyrosequencing. All authors provided intellectual input and A.M. supervised the studies and co-wrote the manuscript.

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Figures

Figure 1

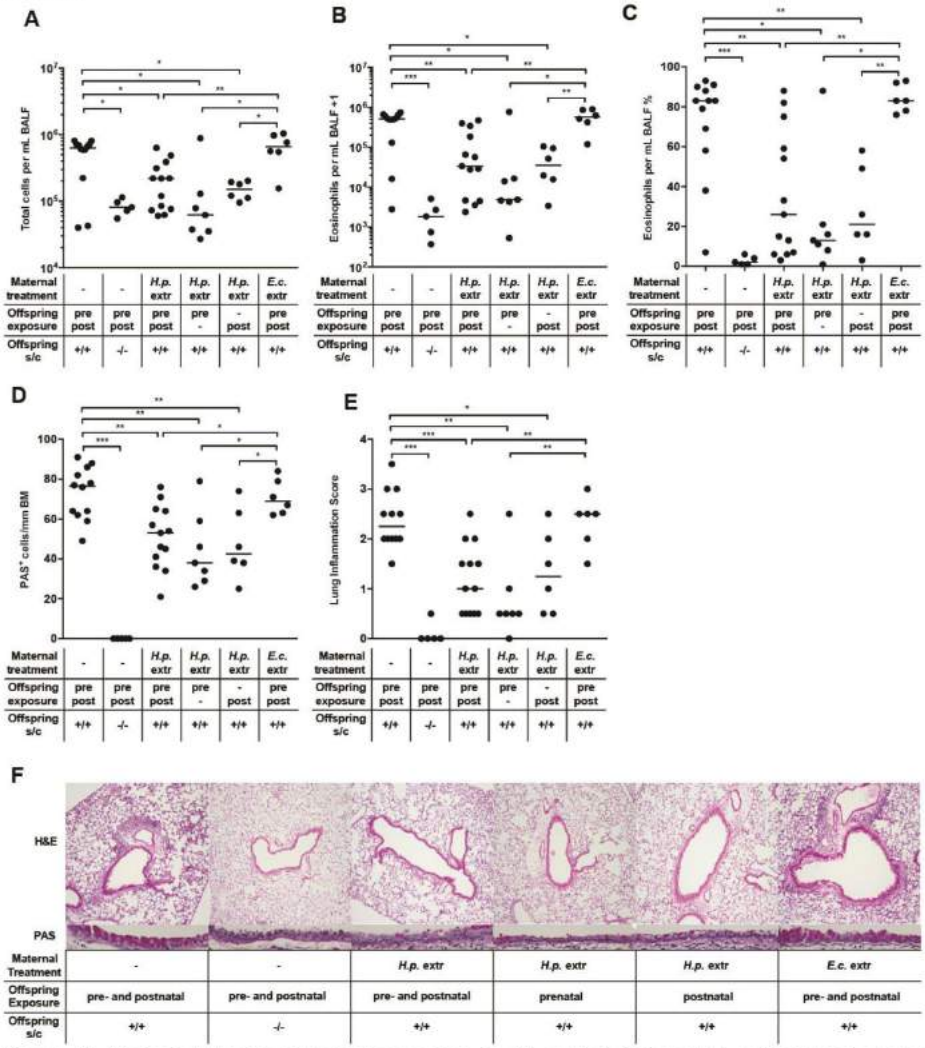


Figure 1. Perinatal trans-maternal exposure to *H. pylori* but not *E. coli* extract protects against house dust mite-induced allergic asthma. Mice were either pre- and/or postnatally exposed to *H. pylori* or *E. coli* extract through 2-3 weekly oral treatments of the dams during pregnancy and/or lactation. Litter swaps were conducted at birth wherever necessary to avoid unwanted exposures. At six weeks of age, the offspring was sensitized and challenged intranasally with house dust mite (HDM) allergen. Negative controls were sensitized and challenged with PBS only. Bronchoalveolar lavage (BAL) leukocytes and eosinophils were quantified at the study endpoint; lungs were fixed, H&E-or PAS-stained and scored with respect to peribroncheolar and perivascular inflammation and PAS$^+$ goblet cell metaplasia. **(A)** Total leukocytes in 1 ml of BAL fluid (BALF). **(B)** Total eosinophils in 1 ml of BALF. **(C)** Eosinophil frequencies in BALF. **(D-F)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. BM, basement membrane. Representative sections are shown in F. In A-E, each symbol represents one mouse. The results were pooled from two independent experiments. Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. * p<0.05, ** p<0.01, *** p<0.001.

Figure 2

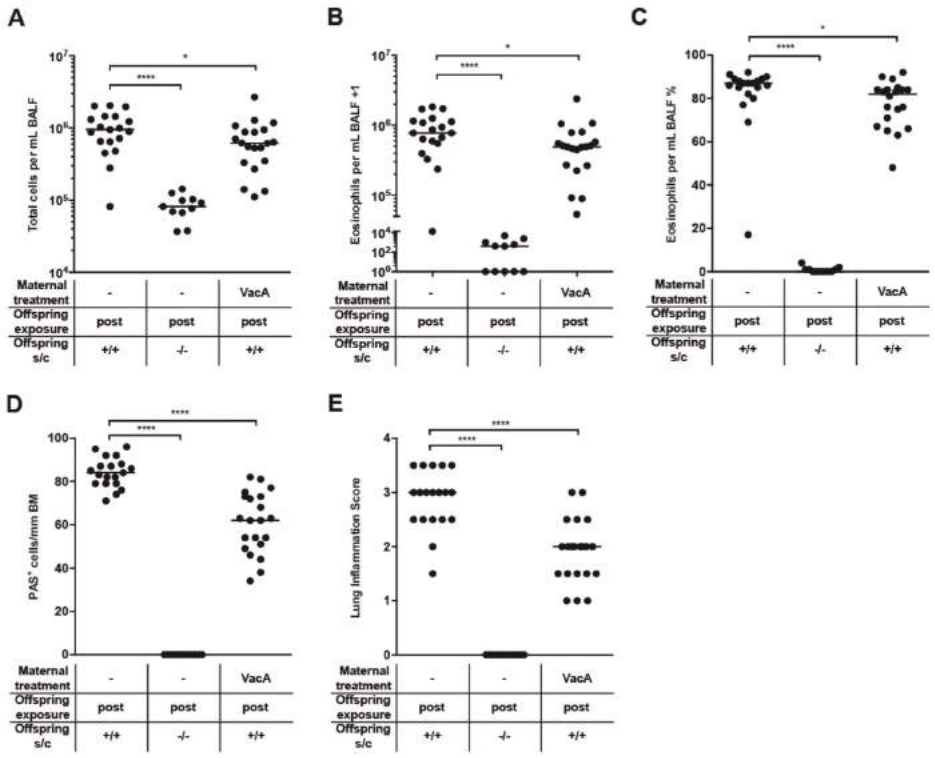


Figure 2. Postnatal trans-maternal exposure to *H. pylori* VacA protects against house dust mite-induced allergic asthma. Mice were postnatally exposed to *H. pylori* VacA through twice-weekly intraperitoneal treatments of the dams with 20 µg purified VacA during lactation. At six weeks of age, the offspring was sensitized and challenged intranasally with HDM allergen. Negative controls were sensitized and challenged with PBS only. Allergic asthma was assessed as described in Figure 1. **(A)** Total leukocytes in 1 ml of BALF. **(B)** Total eosinophils in 1 ml of BALF. **(C)** Eosinophil frequencies in BALF. **(D,E)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. BM, basement membrane. In A-E, each symbol represents one mouse. The results were pooled from three independent experiments. Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Figure 3

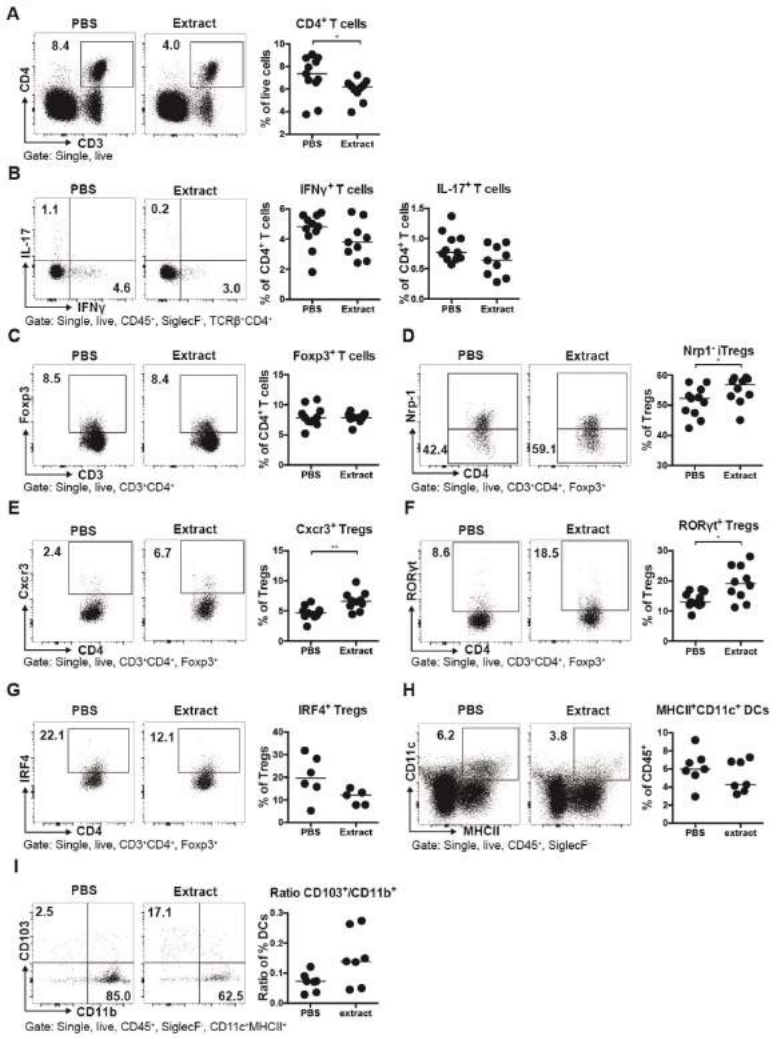


Figure 3. Perinatal trans-maternal exposure to *H. pylori* extract skews lung T-cell responses towards regulatory T-cells. Mice were pre- and postnatally exposed to *H. pylori* extract or PBS through twice-weekly oral treatments of the dams during pregnancy and lactation. At six weeks of age, lamina propria (LP) leukocytes were isolated from the lungs and analyzed by multi-color flow cytometry. For intracellular cytokine staining, LP leukocytes were re-stimulated for 3h with PMA/ionomycin. **(A)** $CD4^+$ T-cell frequencies among all LP leukocytes; representative FACS plots are shown on the left. **(B)** Th1 and Th17 frequencies among all $CD4^+$ T cells, of the mice shown in A. **(C)** $Foxp3^+$ Treg frequencies of the mice shown in A. **(D)** Natural Treg (nTreg; $Nrp\text{-}1^+$) and inducible Treg (iTreg; $Nrp\text{-}1^-$) frequencies among all $Foxp3^+$ Tregs. **(E-G)** Frequencies of $Cxcr3^+$, $ROR\gamma T^+$ and $IRF4^+$ Tregs among all $CD4^+FoxP3^+$ Tregs. **(H)** $MHCII^+CD11c^+$ dendritic cell (DC) frequencies among all lung LP leukocytes. **(I)** Ratios of $CD103^+CD11b^-$ over $CD103^-CD11b^+$ DCs, of all mice shown in H; representative FACS plots are shown on the left. In all panels, each symbol represents one mouse. Data are pooled from two studies (A-F) or show one representative experiment of two to three independent ones (G-I). Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. * $p<0.05$, ** $p<0.01$.

Figure 4

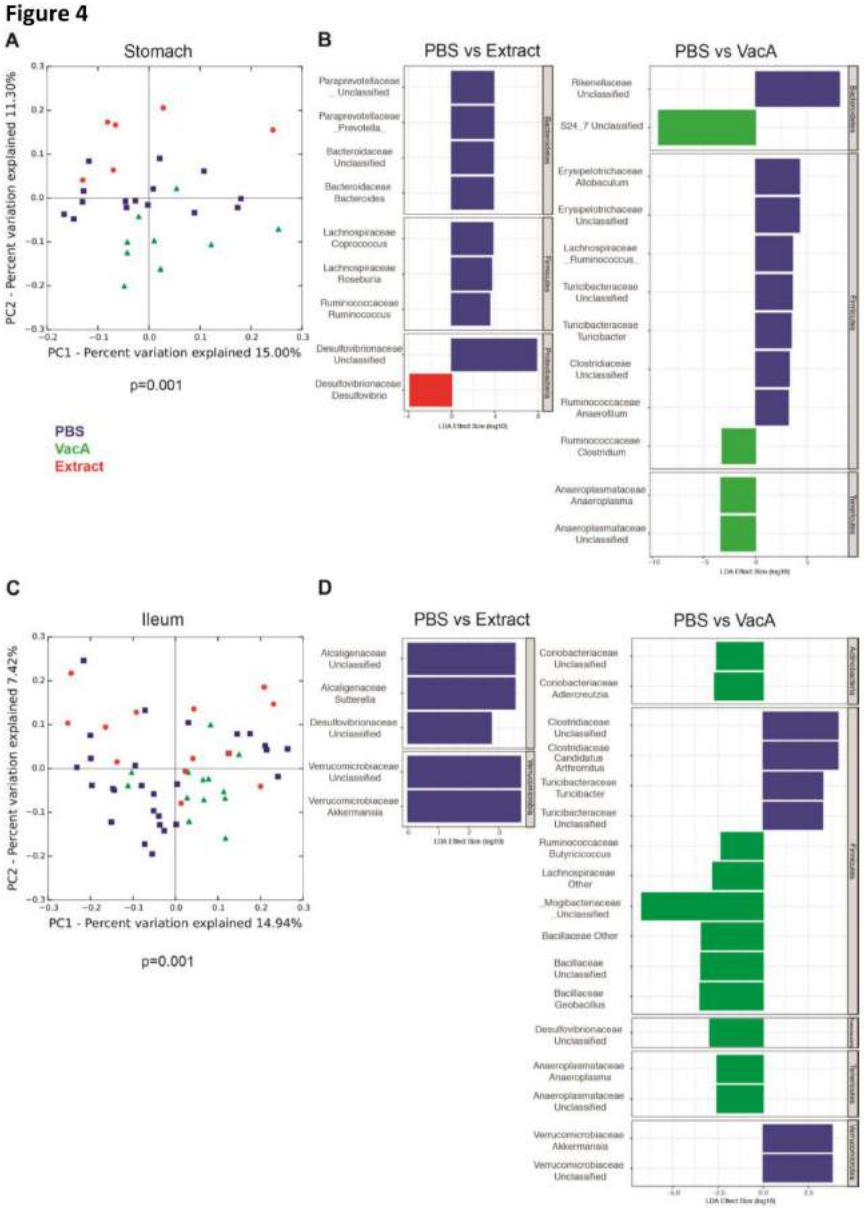


Figure 4. Perinatal trans-maternal exposure to *H. pylori* extract induces shifts in the gastric and ileal microbiota. Mice were pre- and postnatally exposed to *H. pylori* extract, VacA or PBS through twice-weekly oral treatments of the dams during pregnancy and lactation. Gastric and ileal tissue was collected at necropsy and subjected to DNA extraction. The V4 region of the bacterial 16S rRNA gene was amplified and sequenced on the Illumina MiSeq platform. **(A,C)** Unweighted UniFrac distances in a principal coordinate analysis (PCA) plot, where samples were rarefied at 2000 reads for the stomach (A) and ileal (C) tissue. The Adonis test was used to compare community structures between all treatment groups at both sites. **(B,D)** OTUs that were significantly depleted or enriched as a consequence of the indicated treatments, for stomach (B) and ileum (D); LDA scores were generated using LefSE.

Figure 5

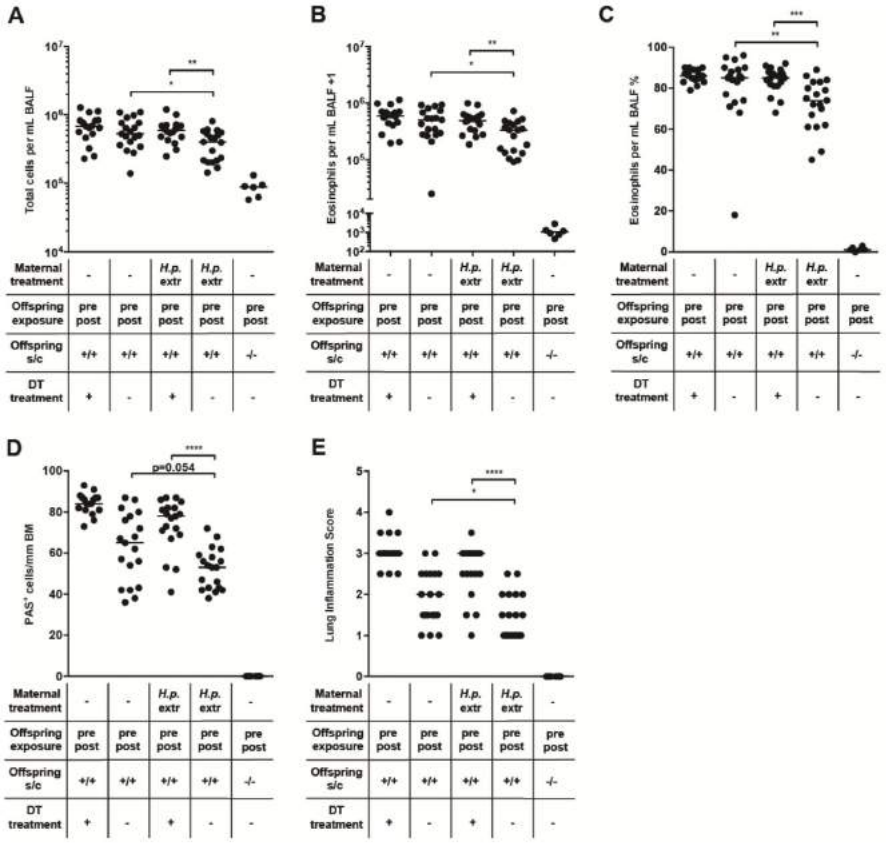
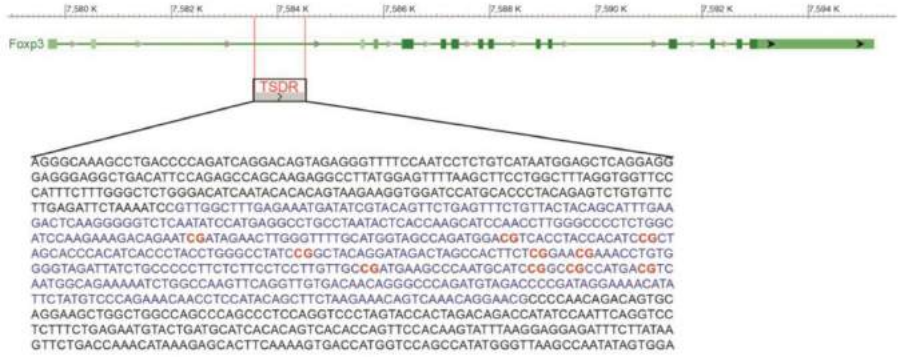


Figure 5. The depletion of regulatory T cells during HDM challenge abrogates asthma protection induced by perinatal trans-maternal exposure to *H. pylori* extract. Foxp3^{DTR} mice were pre- and postnatally exposed to *H. pylori* extract through twice-weekly oral treatments of the dams during pregnancy and/or lactation. At six weeks of age, the offspring was sensitized and challenged intranasally with house dust mite (HDM) allergen. Negative controls were sensitized and challenged with PBS only. Where indicated, mice received a total of four doses (spread across eight days) of 1 µg DT just before and during HDM challenge. Bronchoalveolar lavage (BAL) leukocytes and eosinophils were quantified at the study endpoint; lungs were fixed, H&E-or PAS-stained and scored with respect to peribroncheolar and perivascular inflammation and PAS^{+} goblet cell metaplasia. **(A)** Total leukocytes in 1 ml of BAL fluid (BALF). **(B)** Total eosinophils in 1 ml of BALF. **(C)** Eosinophil frequencies in BALF. **(D,E)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. BM, basement membrane. In A-E, each symbol represents one mouse. The results were pooled from three independent experiments. Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Figure 6

A



B

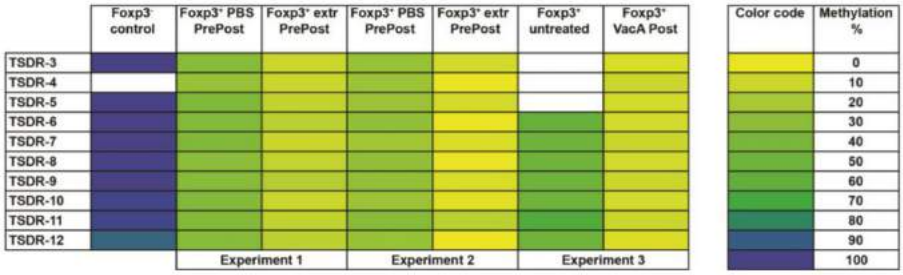


Figure 6. Perinatal trans-maternal exposure to *H. pylori* extract or VacA enriches for regulatory T-cells with a demethylated *FOXP3* TSDR. Mice were pre- and postnatally exposed to *H. pylori* extract or PBS, or postnatally only to VacA, through twice-weekly oral treatments of the dams during pregnancy and lactation (oral treatment with extract and PBS; intraperitoneal treatment with VacA). Foxp3$^+$ Tregs and Foxp3$^-$ CD4$^+$ T-cells were FACS-sorted from the mesenteric lymph nodes and subjected to DNA extraction. DNA was bisulfite converted and subjected to TSDR-specific pyrosequencing. **(A)** Schematic representation of the *FOXP3* locus with the TSDR upstream of the TSS (retrieved using BLAST). The CG-rich region is marked in blue and CG motifs covered by pyrosequencing are labeled in red. **(B)** Color-coded (right panel) methylation pattern of ten CG dinucleotides within the TSDR region. White cells indicate sequences that failed to yield interpretable results due to technical problems. Data are from three independent experiments; cells from five to ten male mice were pooled per treatment group prior to DNA extraction.

Figure 7

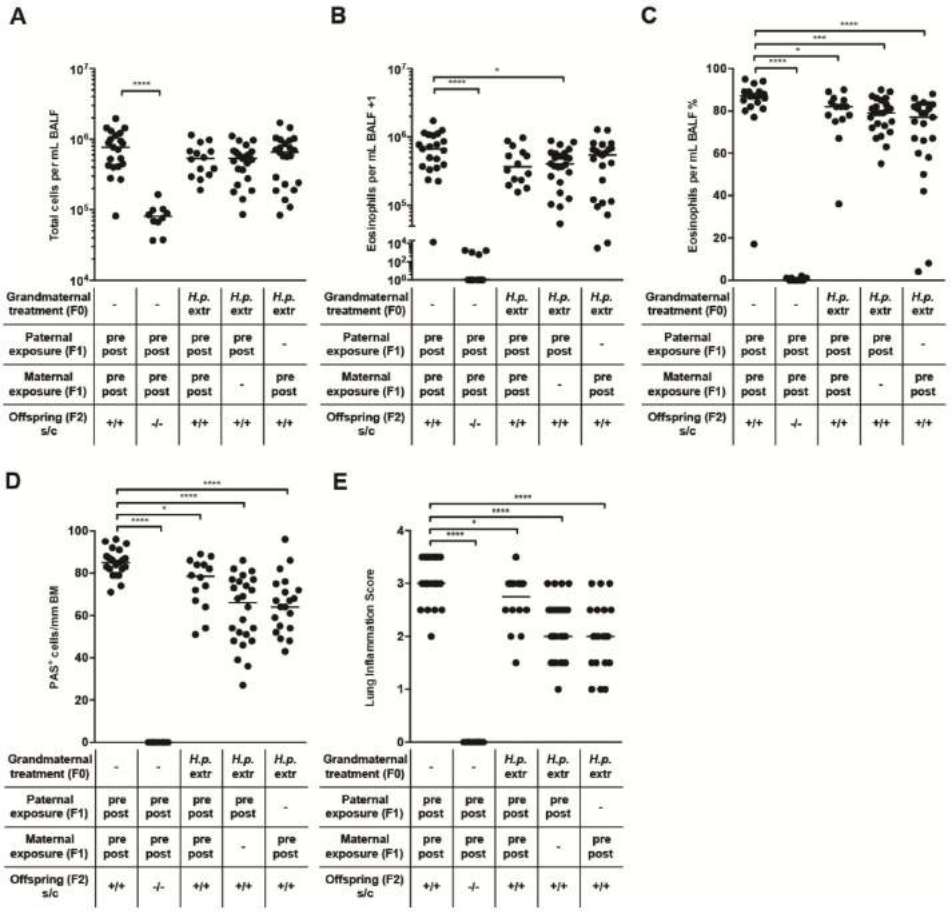
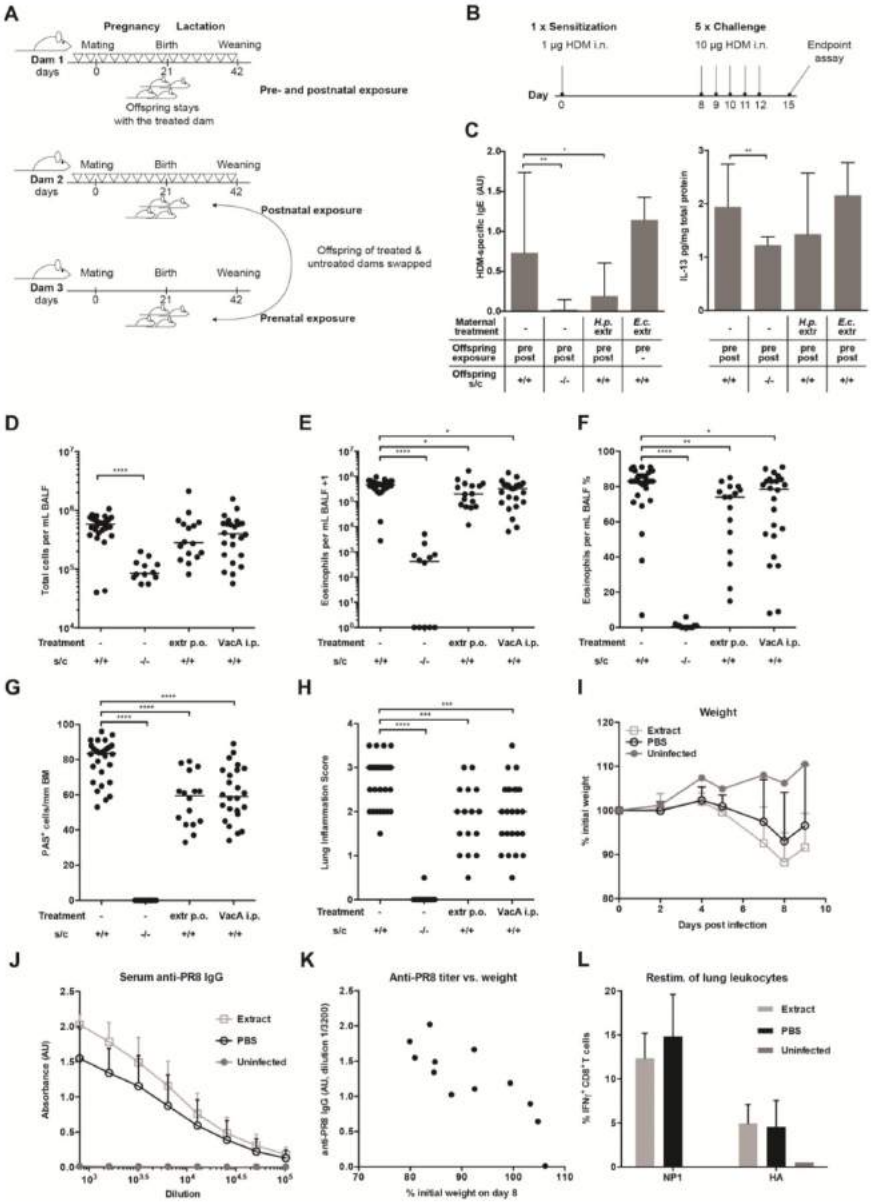


Figure 7. *H. pylori* extract induces inter-generational protection against allergic asthma. F0 dams were subjected to twice-weekly oral gavage with *H. pylori* extract during pregnancy and lactation. Perinatally exposed F1 animals obtained in this manner were bred with each other or with naïve mates. At six weeks of age, F2 progeny were sensitized and challenged intranasally with house dust mite (HDM) allergen. Negative controls were sensitized and challenged with PBS only. Bronchoalveolar lavage (BAL) leukocytes and eosinophils were quantified at the study endpoint; lungs were fixed, H&E-or PAS-stained and scored with respect to peribroncheolar and perivascular inflammation and PAS$^+$ goblet cell metaplasia. **(A)** Total leukocytes in 1 ml of BAL fluid (BALF). **(B)** Total eosinophils in 1 ml of BALF. **(C)** Eosinophil frequencies in BALF. **(D,E)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. In A-E, each symbol represents one mouse. Results were pooled from three independent experiments. Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

Supplemental Figures

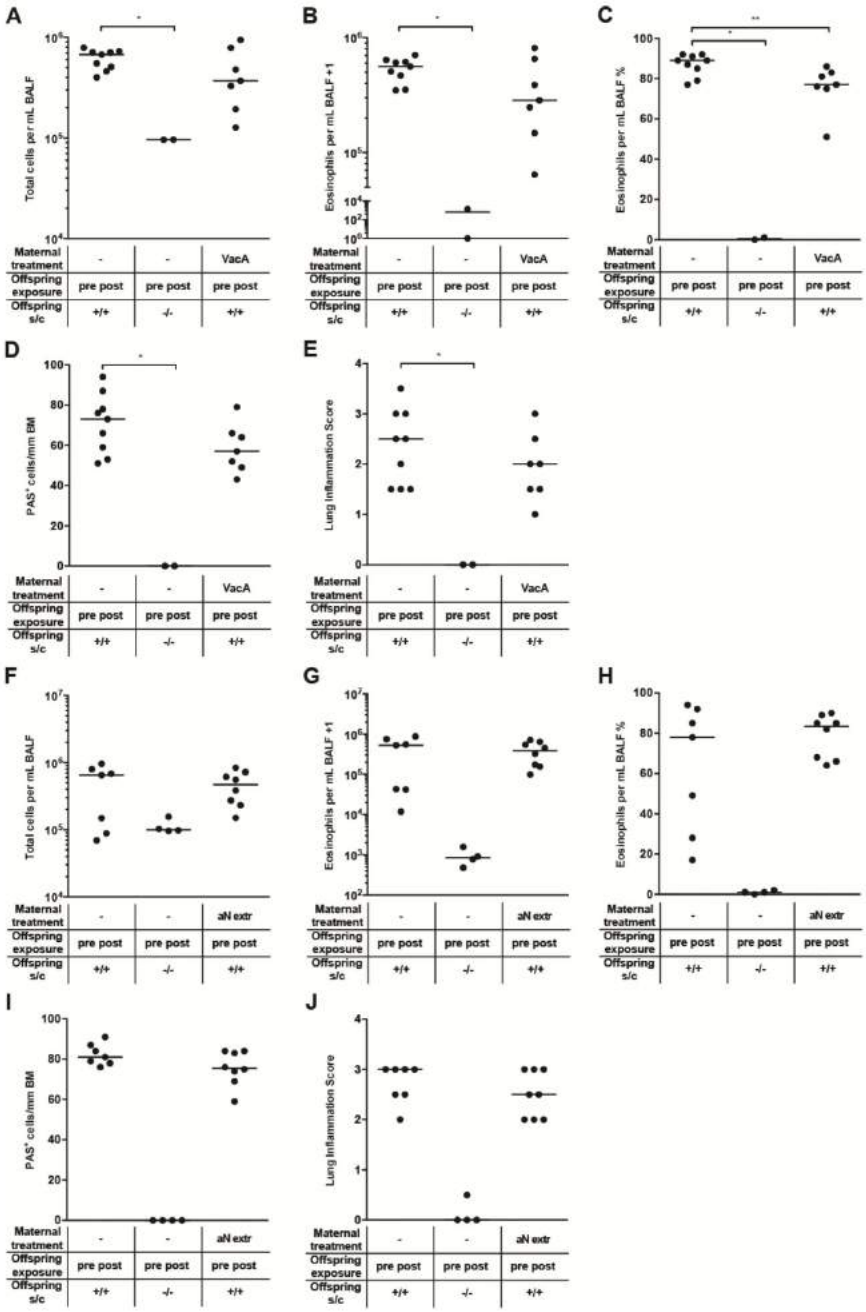
Suppl. Figure 1



Suppl. Figure 1. Perinatal trans-maternal exposure to *H. pylori* protects against house dust mite-induced allergic asthma, but does not induce generalized immunosuppression. Mice were either pre- and/or postnatally exposed to *H. pylori* (or *E. coli*) extract through twice weekly oral treatments of the dams during pregnancy and/or lactation. Litter swaps were conducted at birth wherever necessary to avoid unwanted exposures. At six weeks of age, the offspring was sensitized and challenged intranasally with

house dust mite (HDM) allergen. Negative controls were sensitized and challenged with PBS only. **(A,B)** The schematics in A and B show the general study design (A) and the protocol used throughout this study for inducing allergic asthma with HDM allergen (B). **(C)** HDM-specific IgE, as assessed by serum ELISA, and pulmonary IL-13 production, normalized to total protein content, as assessed by ELISA, of the mice shown in Figure 1. **(D-H)** Mice were treated orally with *H. pylori* extract or i.p. with VacA from day 7 of life onwards until the study endpoint; mice were additionally sensitized and challenged intranasally with HDM allergen as described above. Bronchoalveolar lavage (BAL) leukocytes and eosinophils were quantified at the study endpoint; lungs were fixed, H&E-or PAS-stained and scored with respect to peribroncheolar and perivascular inflammation and PAS^+ goblet cell metaplasia. **(D)** Total leukocytes in 1 ml of BAL fluid (BALF). **(E)** Total eosinophils in 1 ml of BALF. **(F)** Eosinophil frequencies in BALF. **(G,H)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. BM, basement membrane. In D-H, each symbol represents one mouse. The results were pooled from three independent experiments. Horizontal lines indicate medians. **(I-L)** Mice were pre- and postnatally exposed to *H. pylori* extract as described above, and were, at eight weeks of age, infected intranasally with 200 PFU of influenza A virus PR8. **(I)** Weight as plotted in % of initial body weight on the day of infection. **(J)** PR8-specific serum IgG titers, as detected by ELISA on day 9 post infection. The absorbance at 450 nm-620 nm is plotted for serial dilutions of serum. **(K)** High weight loss on day 8 post infection (plotted as % initial weight) is directly correlated with high levels of anti-PR8 IgG in the serum on day 9 post infection (plotted as absorbance at a dilution of 1:3200) for both experimental groups. **(L)** IFN-γ production by $CD8^+$ T-cells, as detected by intracellular cytokine staining and flow cytometric analysis of leukocytes that had been isolated from the lungs of infected mice on day 9 post infection and re-stimulated *in vitro* with PR8-specific peptides $NP1_{366–374}$ (dominant epitope) and $HA_{211-225}$ (sub-dominant epitope) or the irrelevant control peptide $OVA_{257-264}$. The mean (+SD) percentage of IFN-γ-producing $CD8^+$ T cells for each specific epitope is plotted, after subtraction of the percentage of non-specific response to the irrelevant control peptide. PBS and extract-treated groups each consist of 5 or 6 mice respectively. Representative data of one uninfected animal is plotted alongside the infected mice. An unpaired Mann-Whitney U test was used for calculation of p-values throughout. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

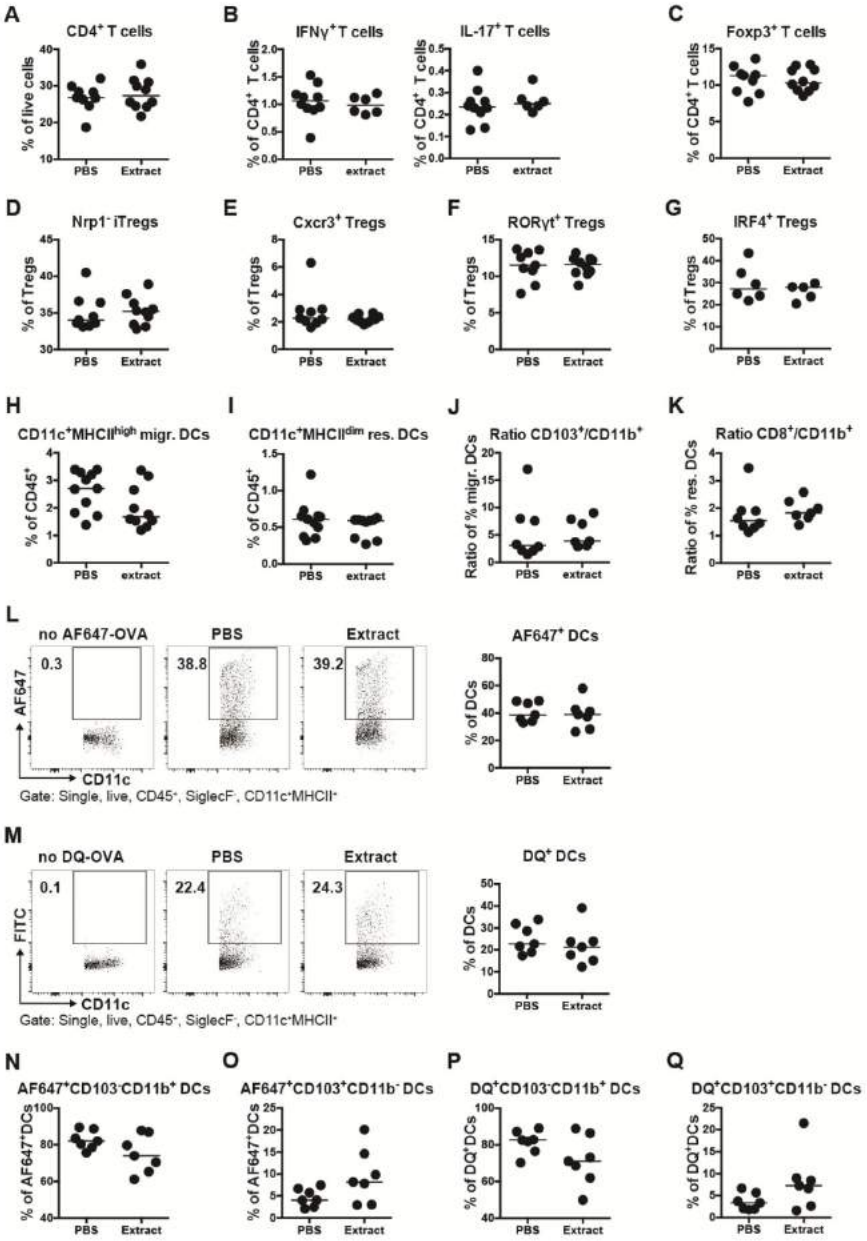
Suppl. Figure 2



Suppl. Figure 2. Pre- and postnatal trans-maternal exposure to *H. pylori* VacA protects against house dust mite-induced allergic asthma. Mice were pre- and postnatally exposed to *H. pylori* VacA through twice-weekly oral treatments of the dams with 20 µg purified VacA during pregnancy and lactation. At six weeks of age, the offspring was sensitized and challenged intranasally with HDM allergen. Negative

controls were sensitized and challenged with PBS only. Allergic asthma was assessed as described in suppl. Figure 1. **(A)** Total leukocytes in 1 ml of BALF. **(B)** Total eosinophils in 1 ml of BALF. **(C)** Eosinophil frequencies in BALF. **(D,E)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. BM, basement membrane. The results in A-E are all from one experiment. Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. * $p<0.05$, ** $p<0.01$. **(F-J)** Female mice were treated orally with *H. pylori* extract from day 7 of life onwards. At seven weeks of age, the treatment was discontinued and females were bred with naïve males to obtain offspring which, at six weeks of age, was sensitized and challenged intranasally with HDM allergen. **(F)** Total leukocytes in 1 ml of BALF. **(G)** Total eosinophils in 1 ml of BALF. **(H)** Eosinophil frequencies in BALF. **(I,J)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. The results in F-J are all from one experiment.

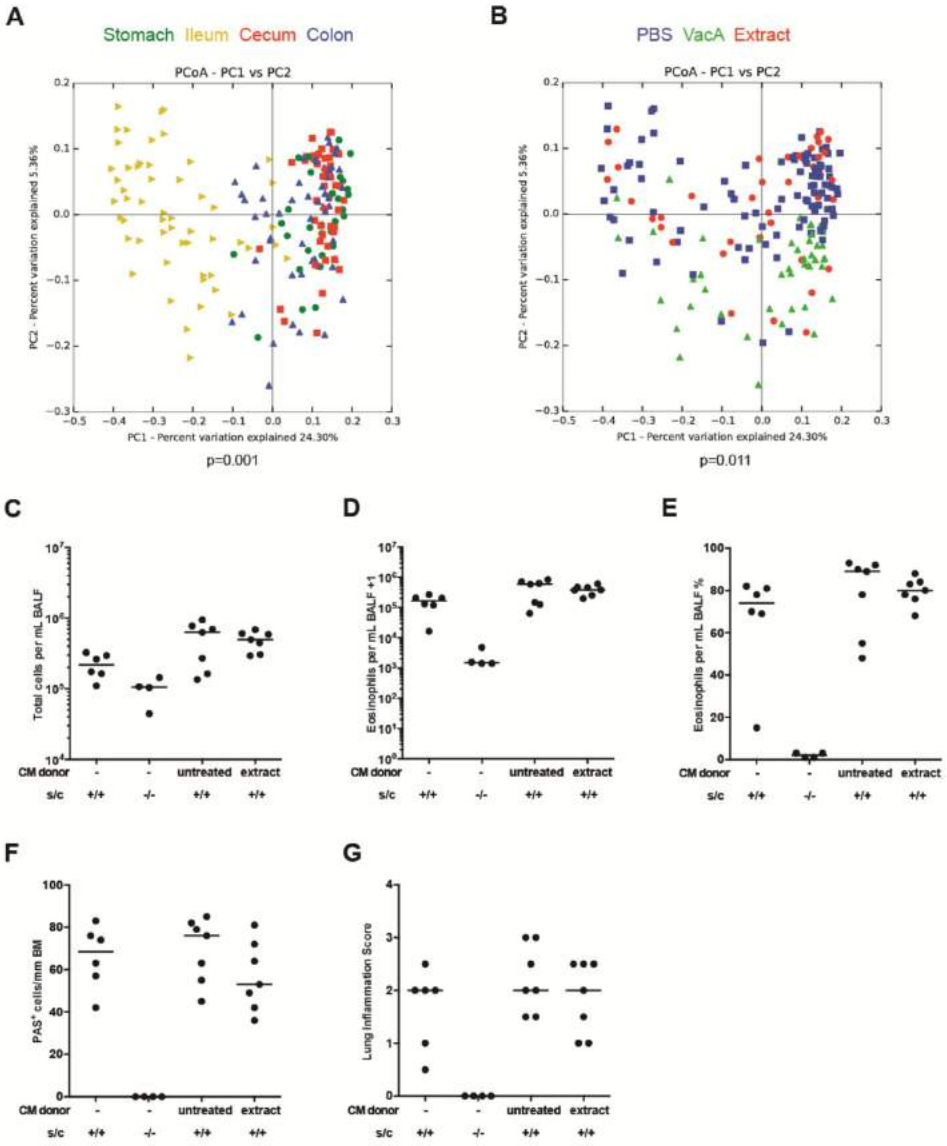
Suppl. Figure 3



Suppl. Figure 3. Perinatal trans-maternal exposure to *H. pylori* extract has no effect on regulatory T-cell populations in the mesenteric lymph nodes or on antigen processing and presentation by dendritic cells. Mice were pre- and postnatally exposed to *H. pylori* extract or PBS through twice-weekly oral treatments of the dams during pregnancy and lactation, as described in Figure 3. At six weeks of age, lamina propria (LP) leukocytes were isolated from the lungs and, along with MLN T-cell and DC populations, were analyzed by multi-color flow cytometry. For intracellular cytokine staining, leukocytes were re-stimulated

ex vivo for 3h with PMA/ionomycin. **(A)** $CD4^+$ T-cell frequencies among all live MLN cells. **(B)** Th1 and Th17 frequencies among all MLN $CD4^+$ T cells, of the mice shown in A and Figure 3. **(C)** $Foxp3^+$ Treg frequencies in the MLNs, of the mice shown in A and Figure 3. **(D-G)** Frequencies of the indicated Treg subsets, among all MLN $Foxp3^+$ Tregs ($Nrp1^-$, $Cxcr3^+$, $ROR\gamma t^+$ and $IRF4^+$). **(H,I)** Frequencies of $CD11c^+MHCII^{high}$ migratory DCs and of $CD11c^+MHCII^{dim}$ resident DCs, among all $CD45^+$ MLN leukocytes. **(J)** Ratios of $CD103^+$ over $CD11b^+$ migratory DCs. **(K)** Ratios of $CD8^+$ over $CD11b^+$ resident DCs. Data in A-K are pooled from two to three independent experiments, with the exception of G, which represents a single experiment. **(L-Q)** Perinatally extract-exposed mice were intranasally challenged with 50 µl DQ-OVA (800 µg/ml) and AF647-OVA (800 µg/ml) approximately 15 hours prior to euthanization and pulmonary leukocyte isolation. **(L,M)** Frequencies of $AF647^+$ (L) and $FITC^+$ DCs (indicating processed DQ-OVA, which fluoresces in the FITC channel, M) among all $CD11c^+$ DCs. **(N-Q)** Frequencies of $CD103^-CD11b^+$ and $CD103^+CD11b^-$ DC subsets among all $AF647^+$ DCs (N,O) or all DQ^+ ($FITC^+$) DCs (P,Q). Data in L-Q are from one experiment, but representative of two independently conducted ones.

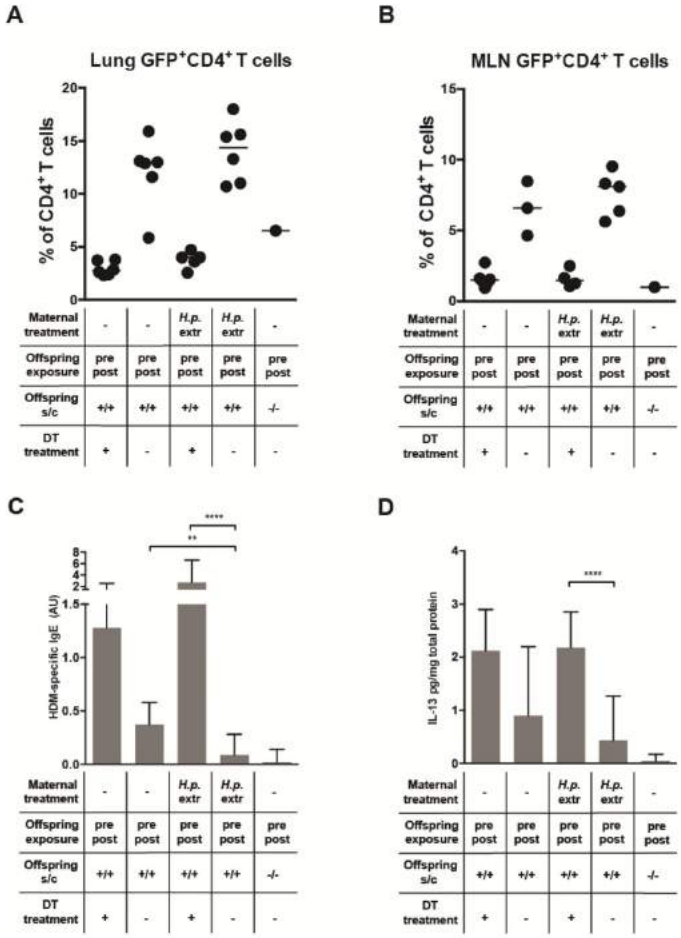
Suppl. Figure 4



Suppl. Figure 4. Perinatal trans-maternal exposure to *H. pylori* extract or VacA induces changes in the microbiota, which however do not confer protection against allergic asthma upon cecal microbiota transfer. Mice were pre- and postnatally exposed to *H. pylori* extract, VacA or PBS through twice-weekly oral treatments of the dams during pregnancy and lactation. Gastric, ileal, cecal and colonic tissue was collected at necropsy and subjected to DNA extraction. The V4 region of the bacterial 16S rRNA gene was amplified and sequenced on the Illumina MiSeq platform. **(A,B)** Unweighted UniFrac distances shown in a principal coordinate analysis (PCA) plot, where samples were rarefied at 2000 reads for all the organs assessed (A) and for all the treatments tested (B). The Adonis test was used to compare community structures between all treatment groups at both sites. **(C-G)** The cecal microbiota (CM) of *H. pylori* extract-

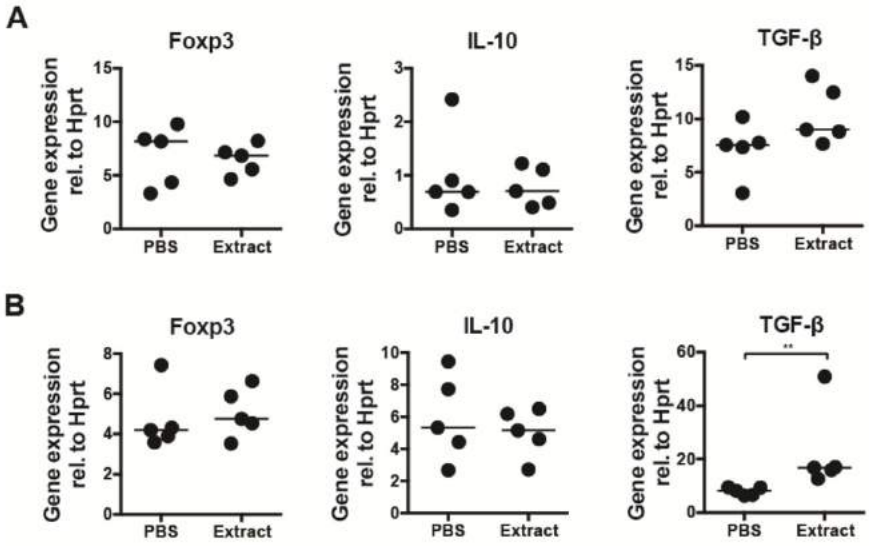
exposed or unexposed adult mice was isolated and transferred into neonates. At six weeks of age, recipients of CM of exposed and unexposed donors were sensitized and challenged intranasally with HDM allergen, alongside mice that had not received CM. Negative controls were sensitized and challenged with PBS only. Allergic asthma was assessed as described in suppl. Figure 1. **(C)** Total leukocytes in 1 ml of BALF. **(D)** Total eosinophils in 1 ml of BALF. **(E)** Eosinophil frequencies in BALF. **(F,G)** Pulmonary inflammation and goblet cell metaplasia, as assessed on stained lung sections. The results in C-G are all from one experiment. Horizontal lines indicate medians.

Suppl. Figure 5



Suppl. Figure 5. The depletion of regulatory T cells during HDM challenge abrogates asthma protection induced by perinatal trans-maternal exposure to *H. pylori* extract. Foxp3^{DTR} mice were pre- and postnatally exposed to *H. pylori* extract through twice-weekly oral treatments of the dams during pregnancy and/or lactation, as described in Figure 5. At six weeks of age, the offspring was sensitized and challenged intranasally with house dust mite (HDM) allergen. Negative controls were sensitized and challenged with PBS only. Where indicated, mice received a total of four doses (spread across eight days) of 1 µg DT just before and during HDM challenge. The Treg depletion efficiency was assessed in pulmonary LP leukocyte preparations as well as the MLNs, and serum HDM-specific IgE levels as well as pulmonary IL-13 production were quantified by ELISA. **(A,B)** Treg depletion efficiency in the lung (A) and MLNs (B), as assessed by FACS of GFP$^+$ (Foxp3$^+$) CD4$^+$ T-cells at sacrifice (i.e. 3 days after the last dose of DT). Note that not all MLNs could be analyzed due to technical reasons. Horizontal lines indicate medians. Data are from one representative experiment of a total of three. **(C,D)** HDM-specific IgE levels in serum and IL-13 concentration in lung homogenates, as determined by ELISA. Bars represent the median with the interquartile range. Data in C and D are pooled from two independent experiments. An unpaired Mann-Whitney U test was used for calculation of p-values. ** p<0.01, **** p<0.0001.

Suppl. Figure 6



Suppl. Figure 6: Expression of Foxp3, IL-10 and TGF-β in pulmonary and MLN-derived, FACS-sorted Tregs. Foxp3$^{eGFP\text{-}DTR}$ mice were pre- and postnatally exposed to *H. pylori* extract through twice-weekly oral treatments of the dams during pregnancy and/or lactation, as described in Figure 6. CD4^{+}GFP^{+} Tregs were sorted from pulmonary leukocyte preparations (A) or MLN single cell preparations (B), and subjected to RNA isolation and qRT-PCR for Hprt, Foxp3, TGF-β and IL-10. Samples were run on a Light Cycler 480 and expression was normalized to the housekeeping gene Hprt. Each symbol represents one mouse. Data are from one experiment. Horizontal lines indicate medians; an unpaired Mann-Whitney U test was used for calculation of p-values. ** $p<0.01$.

4 Discussion

4.1 *H. pylori* and food allergy

4.1.1 Epidemiological correlation

Despite the fact that food allergy represents an important human allergic disorder of increasing prevalence and *H. pylori* is known to be inversely correlated with allergic diseases such as asthma, rhinitis and eczema[313,319,329], studies investigating food allergy and its association with *H. pylori* infection are scarce and controversial. The existing studies are compromised by small sample sizes, heterogeneous populations and non-standardized methodologies[330]. Corrado *et al.* but also others have found a positive correlation of *H. pylori* infection and food allergy, particularly for CagA-positive strains, in children[331,332] and adults[333]. Other studies could not find any association[334] or a negative association[335]. In this latter study a reduced production of allergy mediators (eosinophilic cationic protein and mast cell tryptase) in food allergy patients harboring *H. pylori* relative to non-infected patients was measured. Moreover, a reduced seroprevalence of *H. pylori* in food allergy patients was reported. Such negative associations may clearly be attributed to *H. pylori*'s immunomodulatory abilities to suppress strong host immune responses and skew T polarization towards regulatory T cells. In contrast, positive associations may be explained by a breakdown of the epithelial barrier to food antigens due to a chronic inflammation of the *H. pylori* infected gastric mucosa. Here, we could demonstrate several preventive and protective effects for a range of *H. pylori*-specific treatments in various murine experimental models of food allergy. However, in some experiments, we observed elevated allergen-specific IgE and MCPT-1 levels upon infection but not upon extract or VacA treatment. Nevertheless, we did not find any other evidence for a promoting role of *H. pylori* in the course of food allergy. Therefore, our investigations rather support the studies reporting a negative association of food allergy with *H. pylori* infection.

4.1.2 Different models leading to distinct results

Basically no experimental data addressing the influence of *H. pylori* on food allergy was previously available. This lack might be partially attributed to the fact that no universally accepted food allergy model exists, which would faithfully reflect most aspects of the human disease. In our studies, we have applied four adapted and optimized complementary food allergy models, embracing different routes of sensitization and challenge, two common food allergens, and two different strains of mice to comprehensively investigate the effects of *H. pylori* on clinically relevant hallmarks of food allergy. We found *H. pylori* to be protective in all four murine models of food allergy. Depending on the model, different read-outs were more or less affected by the distinct treatments. In the OVA-model, which was the only model applied using systemic sensitization and subsequent oral challenge, as well as in the orally peanut-sensitized and challenged model, the observed anaphylaxis symptoms were very mild and therefore mice were challenged and observed repetitively. Consequently, T_H2 cytokines in the supernatant of re-stimulated splenocytes and MCPT-1 levels in blood sera were generally lower than measured in other models. In contrast, mice challenged intraperitoneally with peanut extract or in case of the C3H mouse strain, we observed very strong anaphylactic signs, even after only one i.p. or oral (C3H) challenge. On the one hand, this indicates that independent of the sensitization route and allergen used, B6 mice clearly react to an oral food challenge, however, the reaction is much more evident and stronger upon systemic i.p. challenge, which is not the common natural route of food antigen exposure, but still reflects the existence of an allergy against the

food injected. On the other hand, these results demonstrate that the C3H mouse strain is better suited to elicit strong anaphylactic symptoms as well as MCPT-1 secretion upon oral peanut sensitization and challenge. As mentioned in the introduction (*1.1.2.4 Animal models*), these differences are influenced by many factors that are not yet fully understood. C3H mice might react better to an oral peanut challenge due to a TLR4 mutation that blocks intestinal LPS signaling. However, this might only be one part of the underlying mechanisms since BALB/c mice artificially harboring the same mutation still lack responsiveness in such food allergy protocols[200,201]. Further investigations into *H. pylori*'s effects on food allergy induced in C3H mice would certainly be of interest due to the model's robustness and stronger responsiveness, albeit, bearing in mind that mechanistic insights might be difficult to elucidate because of limited availability of genetically manipulated mutant strains.

4.1.3 Effects of neonatal infection on food allergy

In previous years, our lab has convincingly shown that neonatal infection of mice with *H. pylori* leads to protection against allergic asthma, by reducing airway inflammation, airway hyperresponsiveness and goblet cell metaplasia to almost background levels[297,321,325]. Here, we tested neonatal infection in four different models of food allergy. We observed a strong and significant reduction of anaphylaxis-associated symptoms in the OVA-model, and to lesser extent in the orally and i.p. challenged peanut-model. No alleviation of symptoms was detected in the C3H mouse strain peanut-model (data not shown). However, in this model only one experiment was conducted, thus, conclusions have to be drawn carefully. Furthermore, in the OVA model, neonatal infection strongly reduced systemic parameters such as IL-5 and IL-13 in the supernatant of re-stimulated splenocytes and OVA-specific IgG1 in the blood whereas other systemic readouts like allergen-specific IgE and MCPT-1 in serum exhibited an opposite trend. In other models we still observed a minor reduction of MCPT-1 upon infection. However, in the systemically induced peanut food allergy model no decline of T_H2 cytokine secretion from splenocytes and an increase of MCPT-1 upon infection was observed. In the C3H peanut model, the latter two parameters were barely changed upon *H. pylori* infection whereas a slight reduction of allergen-specific IgE and the spleen weight relative to uninfected mice were measured (data not shown). It is difficult to explain these discrepancies between the models and the different read-outs assessed. One pattern that can be deduced from these findings is that no decrease or even an increase in MCPT-1 sera levels upon infection was observed in models that were either systemically sensitized or challenged, but not in models with local oral sensitization and challenge. Furthermore, we could not observe an increase of MCPT-1 levels in the blood of neonatally infected adult mice that were not subjected to a food allergy protocol (data not shown). Hence, neonatal *H. pylori* infection alone is not able to elicit higher MCPT-1 levels and such higher levels seem to be dependent on a systemic allergen trigger accompanied by an infection that activates the mast cells to release more MCPT-1. Furthermore, the anaphylaxis symptoms seem to be more effectively suppressed by *H. pylori* infection in models that induce less strong and severe anaphylaxis. Overall, the beneficial effects of a *H. pylori* infection on food allergy were not as impressive as its impact on allergic asthma outcome. The stronger effects on asthma might be due to the so-called gut-lung axis, an emerging principle proposing a special connection between the mucosal immune systems of the gut and the lung explaining the effects of gastro-intestinal dysbiosis on lung diseases[336].

4.1.4 Effects of neonatal extract treatment on food allergy

As reported for neonatal infection, our lab recently showed that weekly application of *H. pylori*-derived whole-cell extract starting at around day 6 after birth induces stable

protection from asthma in an OVA-induced asthma mouse model[337]. Thus, we sought to elucidate whether extract treatments might also induce protection against food allergy. Indeed, we were able to observe preventive effects in four different models of food allergy using two different allergens and two distinct mouse strains. In the OVA-, the orally challenged peanut-, as well as the C3H-model all assessed read-outs were clearly reduced upon oral extract application, strikingly demonstrating the tolerizing characteristic of *H. pylori*-derived extract in food allergy. For the systemically challenged peanut model a lack of efficacy in reducing anaphylactic symptoms was observed for orally administered extract whereas other assessed hallmarks were still moderately alleviated. Engler *et al.* reported a similar efficacy in asthma protection for i.p. relative to orally injected extract[337]. Consequently, we tested i.p. application of the extract to achieve stronger effects able to suppress the severe systemically induced anaphylaxis. Using this strategy we observed a clear reduction of anaphylactic symptoms, despite not achieving any clear protection in most other read-outs. Systemic extract application appeared to induce a pro-inflammatory response in the spleen, probably boosting the numbers of T cells subsequently re-stimulated *ex vivo* and thus, no protection was detectable when assessing the levels of splenic IL-5 and IL-13 despite observing strongly reduced symptoms. In the same line, it is known that sub-lethal systemic endotoxin application may result in splenomegaly[338]. However, the endotoxicity of *H. pylori*-derived LPS is very low relative to other bacteria[261,262,264]. To further investigate this, one would need to test several dosages of systemic extract application in mice not subjected to a food allergy protocol and subsequently look into the quantity of various effector cell populations in the spleen.

4.1.5 Effects of neonatal VacA treatment on food allergy

Similarly to the above-mentioned *H. pylori* treatments, the *H. pylori*-derived immunomodulatory protein VacA was proven to be sufficient for preventing allergic asthma in an OVA-induced experimental mouse model[337]. Hence, testing the effects of this protein in our food allergy models after observation of protecting effects of live infection and extract treatment was an obvious next step. VacA effectively reduced anaphylactic symptoms in all of the four models tested (data for OVA model not shown), as well as reduced MCPT-1 levels in the blood in most studies. Allergen specific-IgE levels were lower in all models except for the orally sensitized and challenged B6 peanut model. Also inconsistent results were obtained for the measured cytokine concentrations in the supernatant of re-stimulated splenocytes. As shown by Engler *et al.* systemic i.p. application of VacA was sufficient to suppress asthmatic hallmarks, and oral application exhibited also protective effects[337]. The inconsistency of VacA's protective effects on food allergy-related read-outs may therefore partially be attributed to the application route, which may define the grade of efficacy of VacA by making it better or less accessible to the - depending on the allergy protocol - distinctly affected tissues and cells. Consequently, elucidating the best application route and dosage dependent on the food allergy model used, is the next logical step to confirm the effects of VacA treatment on the course of food allergy.

4.1.6 Tregs play a major role

In this work we could demonstrate that Tregs increase in quantity but also upregulate the expression of FoxP3, IL-10 and TGF-β upon exposure of the mice to *H. pylori* and its products. Similarly, we were here (see below) and previously able to link the protection against allergic asthma to the strong suppressive activity of *H. pylori*-induced Tregs. In recent work we have also shown that the induction of Tregs depends on the virulence factor VacA. *H. pylori* strains lacking a functional VacA were not capable of colonizing the murine stomach persistently and at wild-type level, and VacA mutants

failed to induce suppressive Tregs and to confer protection against asthma. In contrast, purified VacA mimicked most of the beneficial effects of a live infection. Interestingly, the asthma-protective effect of *H. pylori*-derived extract seems not to be dependent on Tregs, as adoptive transfer of Tregs from neonatally infected mice were able to suppress allergic asthma in the recipient mice whereas Tregs from extract treated mice were not able to do so[297,298,321,337]. In line with this finding we also could not observe a clear increase of the percentage of Tregs in the mesenteric lymph nodes of extract treated mice although VacA treatment and infection clearly increased the frequency of these cells. Nevertheless, both, IL-10 and TGF-β expression was elevated upon extract treatment, which hints towards the fact that Tregs might play an important role also for extract treatment.
Here, we additionally found an epigenetic correlate of the allergy-preventing activity of Tregs from an *H. pylori*-exposed host. The Treg specific demethylated region (TSDR) of Tregs sorted from mesenteric lymph nodes exhibited lower CpG island methylation patterns upon neonatal infection, extract and VacA treatment relative to untreated control mice. This region essentially controls the stability of FoxP3 expression, hence, demethylation enhances stable lineage differentiation and functionality[129,131,132]. The here detected altered epigenetic signature of Tregs derived from *H. pylori* exposed mice, clearly reflects the immunomodulatory effects we observed upon *H. pylori* infection, extract and VacA treatment, that eventually resulted in protection from food allergy, previously shown for allergic asthma. Furthermore, the fact that VacA alone is able to induce all of the afore-mentioned Treg-associated effects, demonstrates that the strong immunomodulatory properties of *H. pylori* are mediated, at least in part, by the VacA protein.

4.2 *H. pylori* protects against allergic asthma

4.2.1 Neonatal extract tolerization requires BATF3, IL-10, IL-18 but not Tregs

We could show (see results, Engler *et al.*[337]) that intragastric or intraperitoneal weekly administration of *H. pylori* whole cell extract starting on day 6 after birth efficiently prevents allergic asthma development in mice. Thereby, the treatment circumvents the carcinogenic properties of a live infection but still harbors the bacteria's beneficial tolerogenic effects. Interestingly, the protection was as protective as live infection with *H. pylori*, and was, as reported for the infection[321], most effective if started at the neonatal age. This differential susceptibility of neonates and adults may be due to the immature neonatal immune system harboring higher Treg/Teffector cell ratios and Treg-prone responses to foreign antigens[339]. Although our lab could show that the tolerizing effects of a neonatal infection on asthma are highly dependent on *H. pylori*-induced Tregs[297,321], extract treatment-dependent protection seems to be rather independent of this cell type. Adoptive transfer of Tregs from neonatally infected mice to naive recipients protected against asthma in contrast to the same transfer using Tregs from extract-treated mice, which was not protective. Furthermore, depletion of $CD25^+$ Tregs by applying a depleting antibody against CD25 did not affect the extract-induced protection. Accordingly, *H. pylori* extract treatment, in contrast to live infection, of bone marrow derived DCs failed to promote the expression of FoxP3 in cocultured naive T cells, which suggests a Treg-independent mechanism for tolerance induction in case of extract treatment. Indeed our lab could show in earlier research that *H. pylori*-specific tolerance is a consequence of tolerogenic reprogramming of DCs by the bacteria. Here, Engler *et al.* could demonstrate that preferentially $CD103^+CD11b^-$ DCs were recruited to protected lungs and that protection was dependent on this population. Mice lacking the transcription factor BATF3, which drives the development of $CD8\alpha^+$ lymphoid tissue-

resident DC lineages and of the closely related $CD103^+CD11b^-$ DC lineages in various tissues including the lung, intestine and skin, were not responsive to protection conferred by *H. pylori* extract application or live infection[337]. We further could demonstrate that lymph node derived DCs from mice lacking BATF3 are unable to produce IL-10 upon exposure to *H. pylori* extract *ex vivo* whereas wild-type bone marrow or lymph node derived DCs secrete IL-10 upon extract treatment dependent on TLR2 and the downstream adaptor protein Myd88[337]. Additionally, blockade of IL-10 signaling by means of an anti-IL-10R antibody abrogated the protection against asthma and transgenic CD11c-Cre-IL-$10^{fl/fl}$ mice lacking IL-10 specifically in all CD11c-expressing cells exhibit diminished protection. Thus, IL-10 is crucial for extract induced asthma protection and the major source of this cytokine in this context seems to be CD11c-expressing cells, which is further confirmed by the fact that IL-10 secretion by ovalbumin-restimulated CD11c-Cre-IL-$10^{fl/fl}$ total lung cells is diminished by half relative to the wild-type counterpart.
Oertli *et al.* have shown that DC-derived IL-18 is important for Treg induction and that *H. pylori*-infected $IL18^{-/-}$ and $IL18R^{-/-}$ mice do not benefit from protection against asthma[297]. Although Tregs seem not to play a major role in the extract-conferred protection against asthma, Engler *et al.* show here, that $IL18R^{-/-}$ mice fail to build up a protection against asthma upon extract treatment. Thus, IL-18 must play a crucial role for extract-mediated tolerance induction beyond the direct involvement of Tregs. Recently, our lab could further confirm the importance of IL-18 by elucidating the involvement of the TLR2/NLRP3/IL-18 axis in *H. pylori*-induced asthma protection[325]. We could show that *H. pylori*'s urease B subunit (UreB) initiates the TLR2 dependent expression of NLRP3, which leads to the assembly of the NLRP3-inflammasome and subsequent production of IL-1β and IL-18 promoting tolerance and protection against asthma[325,328].
In conclusion, our lab could show here and previously that *H. pylori* mediates immune tolerance and protection against OVA-induced asthma by induction of IL-10 derived from BATF3-dependent DCs and IL-18 via the TLR2/NLRP3 axis. This either promotes Tregs or in case of extract treatment may directly influence T effector responses.

4.2.2 Advantages of the HDM and the OVA models

As already discussed in the introduction (*1.1.1.4 Animal models of allergic asthma*), the HDM-induced allergic asthma mouse model harbors a few advantages over the OVA-induced model. Firstly, the HDM model does not require any additional adjuvants besides the HDM-extract itself due to its intrinsic immunogenic properties probably attributed to 19 epitopes[340] and to the house dust mite's own microbiota containing many bacterial and fungal products such as LPS and β-glucan[161-165], whereas the OVA-model usually relies on an adjuvant like aluminum hydroxide. Secondly, HDMs are the main allergens responsible for allergic asthma in humans, which endows the model with more translatability relative to the OVA allergen, which is not known to cause asthma in humans. Furthermore, the OVA model used here and previously in our lab, consists of two sensitizations with a gap of two weeks in-between, another two weeks later, challenge on three consecutive days is started before mice are sacrificed. In contrast, the HDM model employed here, consists of only one intranasal sensitization, and eight days later mice are subjected to five challenges before the endpoint on day 15, which renders the model much shorter than the OVA model.
Using the HDM model we observed here and for other projects a more robust positive to negative control ratio relative to the OVA model. Moreover, eosinophilia was more pronounced in the HDM model resulting in 10^5-10^6 eosinophils counted per mL BALF whereas in the OVA model only 10^4-10^5 were measured on average. Interestingly, in our hands, lung inflammation and goblet cell metaplasia seems to be comparable between

the two models[325,337]. Regarding the protection against asthma conferred by *H. pylori*, Koch *et al.* could show that a neonatal infection induces significantly lower eosinophilic lung infiltration, lung inflammation, goblet cell metaplasia and HDM-IgE serum levels, and thus, is protective also in the HDM model as previously shown for the OVA model[325]. Likewise, we demonstrate here, that neonatal extract and VacA administration diminishes all assessed hallmarks of asthma in the HDM model to a similar extent as observed in the OVA model although mice are slightly less protected, probably due to the generally higher severity generated by the HDM model. One drawback of the HDM model was the fact that our lab was not able to measure elevated IL-5 secretion from lung single cell preparations upon re-stimulation with HDM, and base line IL-5 secretion was increased in contrast to the OVA model. The larger number of eosinophils or the presence of innate lymphoid 2 cells (ILC2s), which both secrete IL-5 independently of the presence of an antigen, might be responsible for this discrepancy[341,342]. However, we were able to establish further read-outs in the HDM asthma model, such as the assessment of HDM-specific IgE in blood serum and of the IL-13 protein concentration in lung homogenates. To further extend the model, recently published protocols for re-stimulation of mediastinal lymph node single cell preparations and flow cytometric analysis of these cells might be worth establishing, to more accurately evaluate the T cell response[156]. Furthermore, intratracheal instead of intranasal sensitization might lower the variability of the HDM model.

4.2.3 Transmaternal exposure to *H. pylori* prevents asthma development in progeny

4.2.3.1 Transmaternal extract and VacA exposure is highly protective

Early life acquisition of *H. pylori* has been shown to be protective against allergic asthma in several epidemiological and experimental studies, especially in children and young adults or mice infected during the so called neonatal window of opportunity[51,297,313,321,325]. Furthermore, as discussed above, experimental murine models could extensively show that neonatal exposure to *H. pylori* derived extract or purified VacA confers protection against allergic asthma and food allergy[337]. Here we set out to extend these findings by exploiting the effects of prenatal exposure to *H. pylori* during pregnancy, transmaternal postnatal exposure during lactation or the combination of both. To omit the risk of vertical transmission of the infection from dam to offspring, which is deemed to be one of the major routes of *H. pylori* transmission in humans[322], we instead focused on treatments with *H. pylori* extract and VacA. We found that oral extract treatment during pregnancy and lactation significantly reduced all assessed hallmarks of allergic asthma in the offspring, irrespective whether the pups were exposed only pre-, only postnatally, or both. These effects were mimicked when the offspring was exposed pre- and postnatally to oral VacA treated or to intraperitoneally postnatally VacA treated mothers. Strikingly, the degree of protection was comparable to direct neonatal treatment with either extract or VacA.

In contrast to the concept of direct probiotic treatment and exposure to a diversified microbiota embracing many scientific reports and clinical studies, such transmaternal (exclusively via the mother) asthma-protective effects have not yet extensively been tested in experimental models. However, in line with our findings here, it has been postulated that early life and, probably more effectively, prenatal exposure to certain microbes is particularly protective against allergic diseases. In humans, children of mothers exposed to farming environments during pregnancy had a lower risk for allergy development[24,25]. Additionally, Conrad *et al.* could demonstrate that maternal prenatal intranasal exposure to the nonpathogenic cowshed-derived bacterium *Acinetobacter lwoffii F78* protects against the development of experimental asthma in the progeny[343].

Moreover, clinical studies have shown that prenatal supplementation of the mother with *Lactobacillus* and *Bifidobacterium* prevents sensitization to common food allergens and reduces the incidence of atopic eczema in early childhood[4]. Systematic reviews and meta-analyses propose that a combination of pre- and postnatal probiotic supplementation is most efficacious in preventing the development of infant eczema and atopic eczema[344-347], yet no convincing data on beneficial effects on later-onset immunological disorders such as wheezing or allergic asthma have been reported to date[348-351]. This lack of efficacy might be partly attributable to the time point of treatment start, which is often late in the last trimester of pregnancy or postnatally[352]. Increased efficacy has been found for initiating the intervention at the beginning of the second trimester[353].

Since we observed similar protection for pre- and postnatal exposure, we suggest that protective factors are transmitted or induced via placental blood exchange and/or the milk in case of postnatally-induced protection. By transiently colonizing germ-free pregnant mice Ganal-Vonarburg *et al.* could recently show that the offspring's immune system is affected depending on transmission of maternal antibodies that retain microbial molecules during pregnancy and in milk[354]. The underlying mechanisms of *in utero* and postnatal transmission of *H. pylori*-mediated protection might be related or completely distinct; this remains to be elucidated.

Interestingly, extract derived from an *E. coli* strain did not induce protection against asthma in our experiments, indicating that we observed *H. pylori*-specific and not purely endotoxin/LPS-dependent effects and that probably only strong immunomodulators like *H. pylori* are able to mediate postnatal allergy prevention.

Although we observed strong and consistent dampening of eosinophilic infiltration, goblet cell metaplasia and lung inflammation upon transmaternal *H. pylori* extract or VacA treatment, we measured rather inconsistent and unstable effects on HDM-specific IgE in the blood serum and IL-13 in lung homogenates. This might originate from the inability of transmaternal *H. pylori* exposure to reduce initial sensitization towards an allergen, hence IgE levels are only slightly decreased or it might be caused by technical issues of the self-made, yet not fully optimized ELISA used to assess the allergen-specific IgE levels. In case of IL-13, one could try to re-stimulate lung or mediastinal lymph node cells *ex vivo* to achieve more consistent results in the future.

Hypothesizing that the progeny of extract treated dams might be generally immunosuppressed, in particular in the lungs considering the significant protection from allergic airway disease, we tested their ability to handle a pulmonary influenza A virus infection. To our surprise, the viral infection broke the tolerance and the treated mice seemed not to be impaired in mounting an anti-viral response, clearing the infection and recovering from it.

4.2.3.2 Maternal infection does not protect the offspring against asthma

Although it seemed to be highly likely that infected dams transmit their infection to the offspring making our model not differentiable from a neonatal infection, we tested different infection modalities of the maternal animal. To our surprise, we could not detect *H. pylori* in adult offspring, indicating that in mice *H. pylori* is rather unlikely to be vertically transferred from mother to progeny. However, neither neonatal infection nor adult infection of maternal animals was able to induce robust asthma-protection in the offspring. Out of four studies we observed only once lower hallmarks of asthma upon maternal neonatal infection but not adult infection, opposing three other studies for which no effects could be observed. This inconsistency might have been arisen due to a laboratory animal facility room change after the first study, thus, provoking a slight microbiota change of the mice used in later studies and overriding the initially observed phenotype. Assuming that the extract- and VacA-associated protection is caused by a

direct transmission of these bacterial factors via the placenta or the milk, one could also argue that an infection does not provide a sufficient systemic concentration of such factors in order to reach and modulate the offspring's immune system. This issue certainly deserves further investigations confirming the non-protective effect of a maternal infection and elucidating the transmaternal protective mechanism.

4.2.3.3 Transmaternal exposure to *H. pylori* extract skews lung T-cell responses towards regulatory T-cells required for asthma protection

By applying multicolor flow cytometry of steady state lung and mesenteric lymph node cells harvested from adult mice originating from either extract-treated or mock-treated mothers, we analyzed the T cell and myeloid compartments to identify immune correlates responsible for the differential susceptibility to HDM-induced allergic asthma. In the lungs we found lower frequencies of bulk $CD3^+CD4^+$ T cells and T_H1 and T_H17 effector cells, whereas certain Treg subsets exhibited an increased frequency. $FoxP3^+$ Tregs in general were not altered significantly upon perinatal *H. pylori* extract exposure. In line with these findings, $CD103^+CD11b^-$ DCs were more frequent, which might be the cause of the local expansion of Tregs over T effector cells. $CD103^+$ DCs are known to be dependent on the transcription factor BATF3 and for their potency at promoting $CD8^+$ T cell immunity as well as Treg differentiation through the production of retinoic acid and TGFβ[355-358]. Engler *et al.* could also show that these DCs are crucial for *H. pylori*-mediated immune tolerance. $BATF3^{-/-}$ mice do not benefit from extract-, VacA-, or infection-induced protection against allergic airway inflammation[337]. We conducted two pilot studies to investigate the dependence of transmaternally-induced protection on BATF3-dependent lineages (data not shown) on the parental side. In one study, offspring from treated $BATF3^{-/-}$ mothers bred with wild-type males had an abrogated protection against asthma compared to offspring from treated wild-type mothers bred with $BATF3^{-/-}$ males, clearly indicating a role for BATF3-dependent lineages on the maternal side for inducing the protection in the offspring. However, in the second pilot study this trend could not be reproduced. Hence, which of the various Treg subsets is primed by $CD103^+$ DCs, and whether transmaternal *H. pylori*-mediated protection requires these DCs remains to be determined.

Three Treg subsets were over-represented in the lungs of transmaternally-exposed animals: $Nrp1^-$, so-called iTregs or pTregs, most probably induced in the periphery in a thymus-independent manner, $ROR\gamma t^+$ and $CXCR3^+$ Tregs (consisting of p- and to a lesser extent of tTregs). The T_H1 effector T cell master transcription factor T-bet drives the expression of the chemokine receptor CXCR3, which in turn can be used as a marker for cells that at some stage of their ontogeny expressed T-bet[359]. RORγt is known to be the master transcription factor for T_H17 effector $CD4^+$ T cells[109]. Paradoxically, it became recently apparent that certain activated Tregs express the aforementioned effector $CD4^+$ T cell transcription factors which probably endows them with enhanced suppressive capacity. Elimination of T-bet expressing Tregs, but not T-bet expression in Tregs, leads to severe T_H1-driven autoimmunity. Furthermore, $T\text{-}bet^+$ Tregs quite stably express T-bet and were shown to specifically co-localize with T_H1 effector T cells, inhibiting CD8 and T_H1 T cell activation[359]. These results demonstrate that Tregs' functional heterogeneity is crucial for immunological tolerance and goes in line with our findings of *H. pylori*-induced $CXCR^+$ (T-bet-dependent) Tregs. Our results indicate that despite probably being induced and recruited by specific T effector responses, these specialized Tregs are also able to suppress other immune responses than their specialization would let assume, as already proposed by Gérard Eberl[109]. In our experiments T_H1- and T_H17-Tregs are expanded and might suppress a T_H2 mediated response besides dampening T_H1 and T_H2 immunity characterized by the decreased frequency of $IFN\gamma^+$ and $IL\text{-}17^+$ T cells. Counter-intuitively, T_H2 specific $IRF4^+$

Tregs (probably regulated by the master transcription factor GATA3)[134] are rather decreased upon transmaternal *H. pylori* exposure. This most probably is caused by the aforementioned expansion of T_H1 and T_H17 Tregs at the expense of T_H2 Tregs according to the principle of immunity by equilibrium[109].
Interestingly, in the mesenteric lymph nodes (MLN) we could not entirely reproduce the immunophenotype observed for the lungs. However, we observed similar to the lungs a decrease of migratory DCs and a higher $CD103^+/CD11b^+$ ratio, indicating skewing towards a regulatory over an effector response. The lack of a shift of T_H1, T_H17 or T_H2 Tregs in the mesenteric lymph node as well as in the spleen (data not shown) might be attributable to the assumption that these specialized Tregs are induced and fully differentiated or expanded peripherally in target organs/tissues, which in case of *H. pylori* might be mainly the lungs, probably due to the putative existence of a strong gut-lung axis[336].
Supporting the important role Tregs play in transmaternally tolerized mice, we could demonstrate here, that the systemic ablation of $FoxP3^+$ Tregs just before and during the challenge-phase of the asthma-protocol abolishes *H. pylori*-mediated transmaternally-induced protection. Obviously, the mucus-secreting goblet cell infiltration and the lung inflammation score, as well as other hallmarks were aggravated by the systemic depletion of Tregs, which leads to a distortion of the observed effect. Thus, it would be highly interesting to specifically deplete only T_H1 or T_H17 Tregs, to terminally confirm their importance in our observed phenotype. However, this would first require the design and generation of suitable mutant mouse strains. One initial approach might also be to cross the RORγt-flox mouse with the FoxP3-cre mouse (both available from the Jackson Laboratory), to specifically ablate RORγt expression in Tregs.

4.2.3.4 Transmaternal exposure to *H. pylori* extract or VacA affects the diversity and composition of gastrointestinal bacterial community structures

To elucidate whether perinatal extract or VacA treatment also alters the gastrointestinal microbiome in addition to altering allergy severity and immunological correlates of protection, we conducted a 16S rRNA sequencing of samples harvested from the stomach, ileum, cecum and colon. Beta-diversity analysis showed that samples are clearly segregated by treatment and evidently by anatomical site. Furthermore, for each site, samples significantly agglomerated by the respective maternal treatment (PBS, extract or VacA), this was most pronounced in the stomach and ileum. Linear discriminant analysis effect size revealed that different treatments induce higher or lower abundance of different taxa. In conclusion, maternal exposure to *H. pylori* immunomodulators during pregnancy affects gastrointestinal bacterial community structure much later in the adult progeny. This is most probably a consequence of skewing of steady state immune parameters towards a tolerogenic phenotype (as described above), and not the cause of it, because cecal microbiota transplantation from transmaternally protected adult mice to naive newborns was not sufficient to protect against asthma. However, this preliminary piece of data has to be confirmed by further transplantation or co-housing experiments.
Interestingly, certain taxa that were more or less abundant upon treatment are associated with healthy or diseased states. Perinatal VacA treatment led to the enrichment of *Ruminococcaceae Clostridium* in the stomach, which belong to the Clostridium cluster IV, and notably, atopic patients have been shown to possess lower levels of cluster IV Clostridium species[360]. Moreover, in the ileum perinatal VacA treatment resulted in the depletion of *Clostridiaceae Candidatus Arthromitus* belonging to the group of segmented filamentous bacteria associated with a high T_H17 response[361-364]. Recently, Kim *et al.* showed that colonization of pregnant mice with segmented filamentous bacteria increases the risk for neurodevelopmental disorders in

the offspring[365], underlining how changes of these gastrointestinal communities may have far-reaching consequences.

4.2.3.5 Transmaternal extract and VacA treatment induce demethylation of the Treg-specific demethylated region (TSDR) in FoxP3$^+$ Tregs

As previously mentioned, the cowshed-derived bacterium *Acinetobacter lwoffii F78* induces transmaternal asthma protection as observed for *H. pylori* modulators described here[343]. The effects of *Acinetobacter lwoffii F78* were dependent on TLR signaling, IL-6, IL-12 and TNFα production in the mother and epigenetically influenced IFNγ production in the offspring, by protecting CD4$^+$ T cells against loss of the activating histone 4 acetylation in the promoter region of *IFNG* upon OVA sensitization. In contrast, reduced histone 4 acetylation was observed at the *IL4* promoter[366]. However, they did not observe any changes of the methylation pattern at these promoters. These results indicate that in pregnancy such environmental-stimuli are able to quickly induce long-lasting epigenetic changes. In our experiments transmaternal *H. pylori* exposure changed the methylation pattern of Tregs within the so-called Treg-specific demethylated region (TSDR). The TSDR is localized within an intronic enhancer region of the X-chromosome-linked *Foxp3* locus and is selectively demethylated in lineage-committed, stable Tregs[367,368]. Analysis of sorted MLN-derived Tregs of offspring from extract, VacA or sham treated mothers revealed a demethylation of all analyzed CpG islands within the TSDR upon different *H. pylori* treatments, indicating that on average Tregs are more stable. Although, in a preliminary experiment we could not detect methylation changes at the *IFNG* promoter, other regions might be differentially methylated upon transmaternal treatment and also histone modification marks might be altered. Further studies are needed to elucidate these putative changes at other tissue sites, which is difficult to assess since more than $3x10^5$ male cells are required for a comprehensive TSDR methylation analysis, which so far has restricted our efforts to lymph nodes. Furthermore, analysis of CD4$^+$FoxP3$^-$ T cells as conducted for the *Acinetobacter*-study would also be of interest. Currently, our lab is working on establishing the assay for transposase-accessible chromatin using sequencing (ATAC-seq), a technique able to reveal chromatin accessibility on a whole-genome scale using as little as 50'000 cells. Using this assay would allow us to identify and have a closer look at all regions which are epigenetically altered, hence chromatin changes are induced, upon transmaternal *H. pylori* exposure. By applying quantitative real time PCR, we could also show that *H. pylori*-specific perinatal treatment induces a modestly higher expression level of TGFβ but not FoxP3 or IL-10 in Tregs sorted from lungs and MLN, further confirming the treatment's impact on Treg quality and not only quantity of certain subsets. Collectively, we conclude that the epigenetic marks leading to stable expression of FoxP3 and potentially to Treg-specific transcript changes, reflect the suppressive effects of transmaternal *H. pylori* treatments on allergic asthma.

4.2.3.6 The decreased susceptibility to allergic asthma by exposure to *H. pylori* extract is intergenerationally transmitted to the F2 generation

The most remarkable discovery we made in the course of our studies was that *H. pylori*-dependent transmaternal protection against experimentally-induced allergic asthma is intergenerationally transmitted to the F2 generation. Our experiments do not rule out the possibility that *H. pylori* extract directly influences the gametes (that later will give rise to the F2 generation) of the developing fetus (F1) *in utero*. In line with that, it was shown that the risk for allergy and asthma inherited from the mother is up to 5 fold higher than the paternal risk, which partly might be explained by parental imprinting or by direct modification of the fetal immune system *in utero*[369-372]. Our lab is currently setting up such breedings, to disclose whether the observed effects are only

transmitted intergenerationally or even transgenerationally to the F3 generation. Interestingly, for crossings that gave rise to the F2 generation, it did not matter for asthma-protection in the F2 offspring, whether only the male, only the female or both originated from a dam that was treated during pregnancy, ruling out any Mendel-like inheritance of asthma-protection (considering that the protection might be linked to the X-chromosome linked *Foxp3* locus). Our findings so far, strikingly demonstrate how microbial exposures strongly affect allergy risk even across multiple generations.

Similar observations were made for tobacco smoke exposure. Pooled analyses from 15 European birth cohorts demonstrated that even exposure of the mother (non-smoking) to only second hand smoke during the pregnancy will lead to an increased risk for wheezing at age 2 years, which is further increased by postnatal second hand smoke exposure and further elevated in children of atopic families[103]. Most strikingly, Li *et al.* could show that not only maternal but also grand maternal smoking during the mother's fetal period may increase the risk for childhood asthma[104]. Furthermore, in a nicotine-induced asthma model in rats, Rehan and colleagues could demonstrate that asthma provoked by nicotine exposure is transgenerationally transmitted to the F3 generation[373]. Additionally, the pace of the development of the allergic epidemic cannot solely be explained by genetic factors, and further hints towards the involvement of environmentally induced epigenetic mechanisms influencing several generations.

5 Concluding remarks

A plethora of epidemiological, clinical and experimental data impressively demonstrates the vast influence of environmental factors ("exposome") on allergy susceptibility. Most of these factors either directly or indirectly shape the gastrointestinal and/or pulmonary microbial community structure and microbiome diversity of the affected individual, which in turn affects maturation of the immune system and therefore disease susceptibility.

H. pylori co-evolved over thousands of years with its host, which allowed it to adapt to the human immune system employing a range of immune evasion and manipulation mechanisms. This enables the bacterium to efficiently colonize the gastric mucosa. We and others could show that *H. pylori* is a fundamental part of the exposome, not only being a pathogen but also beneficially affecting allergic disease outcome, turning this bacterium into a pathobiont (i.e. a symbiont that is able to provoke pathology when specific genetic or environmental settings are changed in the host).

Herein and recently, we could demonstrate in experimental mouse models that neonatal infection leads to robust asthma protection in the adult host in a Treg-dependent manner via the TLR2/NLRP3/IL18 axis. These effects could be mimicked by using *H. pylori*-derived extract and were shown to rely on the immunomodulator VacA. Protection against asthma conferred by *H. pylori* extract was shown to be mediated by BATF3-dependent DCs secreting IL-10. Here, we further extended the protective effects to a range of experimental food allergy models, showing that neonatal infection, extract and VacA treatment prevents, although less efficiently and robustly than in the asthma model, food allergy development. We now further demonstrated for the first time, the beneficial, also Treg-dependent, impact of prenatal and postnatal transmaternal *H. pylori* exposure on asthma susceptibility in the progeny and characterized the associated altered immune correlates such as a decreased frequency of DCs and bulk CD4 T cells and increased frequencies of specialized Treg subsets in the lungs. Furthermore, these effects were linked to shifts in the microbiota composition, as well as the epigenetic signature of Tregs indicating qualitative and/or quantitative differences in the stability and functionality of Tregs. Notably, transmaternal *H. pylori* exposure did not lead to a generalized immunosuppression as an acute infection with influenza A virus was readily controlled by the mice. These observations suggest the suitability of *H. pylori*-derived products such as VacA as potentially preventive drugs against the allergy epidemic, especially if applied early or even prenatally in atopic families. Most strikingly, we observed that the asthma-protective effects were propagated to the second generation without any further treatments, highlighting the importance of a healthy exposome - Of which *H. pylori* constitutes a key player - in shaping allergy risk and severity over several generations.

Further research is required to elucidate the mechanism and routes responsible for tolerance transmission in the first and second generation on the maternal side as well as tolerance induction on the offspring side. It would also be of great interest to test the existence of, and to shed light on a potential phenotype in the F3 generation. Moreover, to identify the genome-wide epigenetic changes involved in the observed phenotype, our lab is currently establishing the ATAC sequencing method. Finally, the here observed ability of pre- and postnatally supplied *H. pylori* to skew the developing immune system intergenerationally towards immune tolerance, might also apply to other constituents of the exposome, thus, opening up many new options for probiotic allergy prevention.

6 Abbreviations

Ag	Antigens
AIT	Allergen immunotherapy
AP1	Activator protein 1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATAC-seq	Assay for transposase-accessible chromatin using sequencing
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BATF3	Basic leucine zipper ATF-like transcription factor 3
Cag-PAI	Cag pathogenicity island
CagA	Cytotoxin-associated gene A
cAMP	Cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2
CCL20	C-C motif chemokine ligand 20
cDC	Conventional DC
CD*XY*	Cluster of differentiation *XY*
CED	Chronisch entzündliche Darmerkrankungen
CLR	C-type lectin receptor
CNS2	Conserved noncoding sequence 2
CREB	cAMP-responsive-element-binding protein
CS	Cesarean section
CT	Cholera toxin
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR3	C-X-C motif chemokine receptor 3
d	Deletion region
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EPIT	Epicutaneous immunotherapy
ETS	Environmental tobacco smoke
FcγRIII/IV	IgG Fc γ heavy chain binding receptor III or IV
FcεRI	IgE Fc ε heavy chain binding receptor I
FoxP3	Forkhead box P3
GATA3	GATA binding protein 3
GGT	Gamma-glutamyl transpeptidase
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
hBD2/3	Human beta-defensin 2 or 3
HDM	House dust mite
HLA	Human leukocyte antigen
HRV	Human rhinovirus
i	Intermediate region
i.p.	Intraperitoneal
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon-γ

IgE/G1/A	Immunoglobulin E, G1 or A
IL-*XY*	Interleukin-*XY*
IL18R	IL-18 receptor
ILC	Innate lymphoid cell
ILC1/2/3	Group 1, 2 or 3 of innate lympoid cells
iNOS	Inducible nitric oxide synthase
IRF3	Interferon regulatory factor 3
IRF4	Interferon regulatory factor 4
iTreg	Induced regulatory T cell (or peripherally derived pTreg)
LAG-3	Lymphocyte-activation gene 3
LPS	Lipopolysaccharide
m	Mid region
M cells	Membrane cells
MAMPs	Microbe-associated molecular patterns
MCPT-1	Mast cell protease 1
MLN	Mesenteric lymph nodes
mSC	Membrane secretory component
Myd88	Myeloid differentiation primary response 88
Mϕs	Macrophages
NCDs	Non-communicable diseases
NFAT	Nuclear factor of activated T cells
NK	Natural killer cell
NLRP3	NACHT, LRR and PYD domains-containing protein 3
Nrp-1	Neuropilin-1
nTreg	Natural regulatory T cell (or thymus derived tTreg)
OIT	Oral immunotherapy
OR	Odds ratio
OVA	Ovalbumin
OX40L	OX40 ligand
PAF	Platelet activating factor
PAI	Pathogenicity island
PAMP	Pathogen-associated molecular pattern
pIgR	Polymeric Ig receptor
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
ROR-γt	Retinoic acid receptor-related orphan receptor-γt
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RSV	Respiratory syncytial virus
s	Signal region
SEB	Staphylococcal enterotoxin B
SIgA	Secretory IgA
SLIT	Sublingual immunotherapy
SMAD3	Mothers against decapentaplegic homologue 3
SNPs	Single nucleotide polymorphisms
STAT5/6	Signal transducer and activator of transcription 5 or 6
TBK-1	Serine/threonine protein kinase-1
TCR	T cell receptor
T_{FH}	T follicular helper cell
TGF-β	Transforming growth factor-beta1
T_H1	T helper 1 cells
T_H17	T helper 17 cells

T_H2	T helper 2 cells
TIEG1	TGFβ -inducible early gene 1
TLR2/4/9	Toll-like receptor 2/4/9
TNFα	Tumor necrosis factor α
TRAP	Traffic-related air pollution
Treg	Regulatory T cell
TSDR	Treg-specific demethylated region
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
UreB	Urease B subunit
VacA	Vacuolating cytotoxin A
VCAM-1	Vascular cell adhesion molecule 1
WHO	World Health Organisation

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9 Curriculum Vitae

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10 Appendix

10.1 *Helicobacter pylori*-specific protection against inflammatory bowel disease requires the NLRP3 inflammasome and IL-18

Research article published in Inflammatory Bowel Diseases, 2015

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Contribution:	I helped perform experiments for figures 1 and 3, and conducted the revision.

ORIGINAL ARTICLE

Helicobacter pylori–specific Protection Against Inflammatory Bowel Disease Requires the NLRP3 Inflammasome and IL-18

Daniela B. Engler, PhD, Irina Leonardi, MSc,† Mara L. Hartung, Msc,* Andreas Kyburz, MSc,* Sabine Spath, PhD,‡ Burkhard Becher, PhD,‡ Gerhard Rogler, MD, PhD,† and Anne Müller, PhD**

Background: The Gram-negative bacterium *Helicobacter pylori* is a constituent of the human gastric microbiota. Chronic infection with *H. pylori* causes gastritis and predisposes to gastric carcinoma but has also been inversely linked to various allergic and chronic inflammatory conditions. In particular, large meta-analyses have documented an inverse association between *H. pylori* infection and the risk of developing ulcerative colitis and Crohn's disease.

Methods: We investigated possible protective effects of experimental *H. pylori* infection and of regular treatment with *H. pylori* extract in 2 mouse models of colitis and in mouse models of type I diabetes and multiple sclerosis. The mechanism of protection was examined in mouse strains lacking specific innate immune recognition pathways and cytokines.

Results: We show here that experimental infection with *H. pylori* and administration of regular doses of *H. pylori* extract both alleviate the clinical and histopathological features of dextran sodium sulfate–induced chronic colitis and of T-cell transfer-induced colitis. High resolution endoscopy of the protected animals revealed the accumulation of large amounts of colonic mucus upon *H. pylori* exposure, which could be attributed to transcriptional activation of the mucin 2 gene. The protection against dextran sodium sulfate–induced colitis was dependent on the NLRP3 inflammasome and interleukin-18 signaling. Other autoimmune diseases, i.e., experimental autoimmune encephalomyelitis and type I diabetes, were not controlled by *H. pylori*.

Conclusions: In summary, we propose here that the immunomodulatory activity of an ancient constituent of the gut microbiota, *H. pylori*, may be exploited for the prevention and/or treatment of inflammatory bowel diseases.

(Inflamm Bowel Dis 2015;21:854–861)

Key Words: chronic intestinal inflammation, microbial immunomodulation, autoimmunity, mucus production, inflammasome activation

Crohn's disease (CD) and ulcerative colitis (UC), collectively referred to as inflammatory bowel diseases (IBDs), develop in genetically susceptible individuals as the result of an inappropriately aggressive immune response to ubiquitous antigens of the normal intestinal microflora. Genetic studies have highlighted the importance of host–microbe interactions and of innate immune recognition of components of the intestinal microbiota in the pathogenesis of these chronic inflammatory disorders.[1] The prevalence of both IBDs has increased in most developed countries during the past century, a trend that has been attributed to changes in diet, antibiotic use, and environmental factors and to changing patterns of intestinal colonization.[2] The biological hallmarks of active inflammatory bowel disease are a pronounced infiltration of innate and adaptive immune cells into the lamina propria and elevated local levels of their cytokine products, especially tumor necrosis factor-α, IL-1β, interferon (IFN)-γ, and Th17 cell-derived cytokines. Approved treatments for IBDs include aminosalicylates or topical corticosteroids for mild disease forms and systemic corticosteroids, immunosuppressive thiopurines, and biologics targeting tumor necrosis factor-α for moderately active or severe disease courses or acute flares.[3] Alternative therapeutic strategies in (pre) clinical development target cytokines involved in IBD pathogenesis (IL-12 and IL-23), the α4β7 integrin involved in lymphocyte homing to the gut, and JAK3 kinase, which is involved in cytokine signaling.[4] Additionally, rather experimental strategies attempt to modulate pathogenic immune responses in models of IBD by the introduction of helminths, such as *Heligmosomoides polygyrus*[5,6] or *Trichuris suis*[7,8] or of probiotics (especially Lactobacillus or Bifidobacterium species or the Escherichia coli strain Nissle).[9]

Chronic gastric infection with the bacterial pathogen *Helicobacter pylori* causes gastritis and peptic ulcer disease[10] and represents the most important risk factor for gastric cancer[11,12] but has also been linked inversely to the risk of developing allergic diseases and IBD in large epidemiological studies and

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meta-analyses.[13–15] We have shown in various models that experimental *H. pylori* infection prevents allergen-induced asthma by inducing tolerogenic DCs, which acquire the ability to direct the differentiation of protective FoxP3$^+$ regulatory T cells.[16–19] We report here that experimental *H. pylori* infection and regular administration of *H. pylori* extract protect against chronic colitis in the dextran sodium sulfate (DSS) and T-cell transfer models of the disease. Protective effects were evident during colonoscopy and upon histological assessment of affected colonic tissues. Efficient protection from the chronic inflammation typical of DSS-induced colitis required innate immune recognition of *H. pylori* by the NLRP3 inflammasome and the subsequent production and secretion of IL-18. NLRP3$^{-/-}$ as well as IL-18$^{-/-}$ and IL-18R$^{-/-}$ mice or mice lacking the downstream adaptor protein MyD88, failed to respond to *H. pylori* extract treatment. The endoscopy approach further revealed that *H. pylori* extract induced the production of large quantities of protective mucus composed of the intestinal mucin 2 (MUC2). Other autoimmune diseases, such as type I diabetes (T1D), and experimental autoimmune encephalomyelitis were not controlled by *H. pylori* infection or *H. pylori* extract administration. In summary, we provide evidence for the efficacy of a new treatment strategy for IBD that exploits the immunomodulatory properties of *H. pylori*, a naturally occurring infection known to be inversely correlated with IBD risk in humans.

MATERIALS AND METHODS

Animal Experimentation

C57BL/6, BL/6.Nlrp3$^{-/-}$, BL/6.MyD88$^{-/-}$, BL/6.IL18$^{-/-}$, and BL/6.IL18R$^{-/-}$ mice were obtained from Jackson Laboratories and maintained in individually ventilated cages under SPF conditions. Eight-week-old mice were subjected to 3 cycles of 5 days each of 2% DSS followed by 1 week-long compound-free intervals. To induce colitis in T-cell–deficient hosts, BL/6.TCRβ$^{-/-}$ mice (Jackson Labs) were intraperitoneally (i.p.) injected with 400,000 naive CD4$^+$CD62L$^+$CD44^{lo} T cells (purified by immunomagnetic isolation, MagCellect Naive T cell kit; R&D). Mice were either orally infected at 7 days of age with 10^8 *H. pylori* PMSS1 as described[20] or received 3 weekly oral or i.p. doses of 200 μg extract of *H. pylori* PMSS1 beginning after the first cycle of DSS treatment or on the day of T-cell transfer. Colon length was measured from the cecum to the distal rectum; colons were dissected longitudinally with one half being embedded for formalin fixation and paraffin embedding and the other being cryopreserved for later RNA extraction. Paraffin sections were stained with Giemsa and examined in blinded fashion on a BX40 Olympus microscope. For induction of experimental autoimmune encephalomyelitis (EAE), C57BL/6 mice were immunized with 200 μg myelin oligodendrocyte glycoprotein fragment 35-55 (MOG_{35-55}) peptide adjuvanted by complete Freund's adjuvant, followed by two 200 ng i.p. injections of pertussis toxin. Spontaneously developing type 1 diabetes was assessed in female nonobese diabetic mice (NOD/ShiLtJ mice, Jackson labs) at 20 weeks of age. For induction of T1D, male NOD mice were i.p. injected with one 5 mg dose of cyclophosphamide at 10 weeks of age and assessed at 20 weeks of age. The pancreas was formalin-fixed and paraffin-embedded, and H&E-stained sections were scored as described below. The inflammation of 30 pancreatic islets was recorded per animal. All animal experimentation was performed in accordance with federal, cantonal, and institutional guidelines and was approved by the Zurich Cantonal Veterinary Authorities.

Histopathological Analysis of Colitis, Insulitis, and Gastritis, and Clinical Scoring of Colitis and EAE

The scoring system first introduced by Asseman et al[21] was used for the quantitative histopathological assessment of colitis. A grade of 0 was given when no changes were observed; grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3, mild-to-moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with ulceration with marked epithelial hyperplasia and mucin depletion; grade 5, marked transmural inflammation with severe ulceration. Colitis endoscopy scores were defined for the following 5 parameters[22]: thickening of the colon: 0, transparent; 1, moderate; 2, marked, 3, nontransparent; changes of the vascular pattern: 0, normal; 1, moderate; 2, marked; 3, bleeding; fibrin visible: 0, none; 1, little; 2, marked; 3, extreme; granularity of the mucosal surface: 0, none; 1, moderate; 2, marked; 3, extreme; stool consistency: 0, normal and solid; 1, still shaped; 2, unshaped; 3, spread. Overall scores were determined by adding all individual scores to reach 0 to 15. For the daily clinical scoring of EAE, we used a previously published procedure[23] as follows: 0, no detectable signs of EAE; 0.5, distal limp tail; 1, completely limp tail; 1.5, limp tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete bilateral hind limb paralysis and partial forelimb paralysis; 4, moribund (mouse completely paralyzed); 5, dead. Food and water were provided in the cage after scores of >1 were reached. H&E-stained sections of pancreas were scored for insulitis based on a previously published system.[24] Four grades of insulitis were distinguished: grade 0, absence of insulitis; 1, peri-insulitis; 2, light infiltration involving <25% of the islet area; 3, heavy infiltration involving >25% of the islet area. The degree of *H. pylori*–induced gastritis/inflammation, atrophy, and hyperplasia was scored on H&E-stained gastric sections based on the modified "Sydney system," as published previously.[25] Briefly, scores for chronic inflammation were 0, none; 1, some infiltrates; 2, mild (few aggregates in submucosa and

mucosa); 3, moderate (several aggregates in submucosa and mucosa); 4, marked (many big aggregates in submucosa and mucosa); 5, nearly the entire mucosa contains a dense infiltrate; and 6, entire mucosa contains a dense infiltrate. Scores for atrophy were 0, none; 1, foci, where a few gastric glands are lost or replaced; 2, small areas in which gastric glands have disappeared or been replaced; 3, 25% of gastric glands lost or replaced; 4, 25% to 50% of gastric glands lost or replaced; 5, 50% of gastric glands lost or replaced; 6, only a few small areas of gastric differentiated glands remaining. For hyperplasia, the scores were 0, none; 1, single glands (next to infiltrate); 2, 1 focal area/1 to 4 crypts (mild); 3, 1 to 3 foci; 4, multiple foci; 5, 50% of glands affected; 6, only few small nonhyperplasic areas.

Preparation of *H. pylori* Extract and Quantification of *H. pylori* Colonization by Colony Count Assay

Helicobacter pylori was cultured in Brucella broth supplemented with 10% fetal calf serum, pelleted by centrifugation and washed once with phosphate-buffered saline. Bacteria were subjected to 3 freeze/thaw cycles and disrupted by 3 passes through a French pressure cell press (Stansted Fluid Power, Cell Pressure Homogenizer) at 30.000 bar. Cell debris was removed by centrifugation and the supernatant was filtered through a 2-μm filter. Protein concentrations were determined by BCA Protein Kit (R&D systems). For the quantitative assessment of *H. pylori* colonization, one section of each stomach was transferred to a tube containing Brucella broth and homogenized with an Ultra Turrax homogenizer (John Morris Scientific Ltd., Chatswood, Australia). Serial dilutions were plated on horse blood plates to determine bacterial loads.

RNA Extraction, Reverse Transcription, and Quantitative PCR

For real-time RT-PCR, total RNA was isolated from 1 longitudinal section of the colon using NucleoSpin RNA II kits (Macherey-Nagel, Düren, Germany) and reversely transcribed using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA). The corresponding cDNA served as a template for real-time PCR performed using the LightCycler 480 SYBR Green I master kit (Roche, Basel, Switzerland). Absolute values of MUC2, CDX-2, TGF-β, IL-17, and IFN-γ expression were normalized to GAPDH expression (conditions: 60°C annealing temperature, 50 cycles). The following primers were used: GAPDH, forward: 5′-GAC ATT GTT GCC ATC AAC GAC C-3′, reverse: 5′-CCC GTT GAT GAC CAG CTT CC-3′; MUC2, forward: 5′-TGCCCAGAGAG TTTGGAGAG-3′, reverse: 5′-CCTCACATGTGGTCTGGTTG-3′; CDX-2, forward: 5′-CTGGGGTTCTGAAACCAAAT-3′, reverse: 5′-CACCATCAGGAGGAAAAGTGA-3′; TGFb, forward: 5′-TG ACGTCACTGGAGTTGTACGG-3′, reverse: 5′-GGTTCATG TCATGGATGGTGC-3′; IFNg, forward: 5′-CATGGCTGTTT CTGGCTGTTACTG-3′; reverse: 5′-GTTGCTGATGGCCTG ATTGTCTTT-3′, IL-17, forward: 5′-GCTCCAGAAGGCCCT CAG A-3′; reverse: 5′-AGCTTTCCCTCCGCATTGA-3.′

Statistics

All statistical analyses were performed using Graph Pad prism 5.0 software (Graph Pad Software, San Diego, CA). The Mann–Whitney test was used for all comparisons except for those in EAE, where 2-way analysis of variance (ANOVA) was used to determine significant differences over time. P values <0.05 were considered significant.

RESULTS

Live *H. pylori* and *H. pylori* Extract Protect Effectively Against the Clinical and Histopathological Features of Chronic Colitis

To assess whether experimental infection with *H. pylori* or regular administration of *H. pylori* extract modulate the severity of DSS-induced colitis, C57BL/6 mice were either experimentally infected with 1 orogastric dose of the mouse-colonizing *H. pylori* strain PMSS1 at 7 days of age or received intragastrically administered *H. pylori* extract (10 mg/kg body weight) 3 times a week. All mice were exposed to three 5-day long cycles of DSS in the drinking water (2% final concentration), each followed by a compound-free interval of 1 week. At the study endpoint, mice were examined by high resolution colonoscopy and assessed with respect to colonic histopathology and colon length. Histopathological evaluation of the colon revealed that all mice of the positive control group had developed moderate-to-severe colitis, as evidenced by the widespread loss of goblet cells and of crypts, accompanied by extensive mucosal and submucosal infiltration of inflammatory cells (Fig. 1A, B). In contrast, *H. pylori*–infected mice exhibited significantly less inflammation and had also undergone less substantial epithelial changes (Fig. 1A, B). Interestingly, a similar beneficial effect was observed in mice that were treated with *H. pylori* extract. Overall, significantly lower histopathology scores were assigned to the mice in the 2 treatment arms exposed to live *H. pylori* or its extract than to the positive controls (Fig. 1A, B). Lower histopathology scores were associated with a decreased expression of the Th1 and Th17 cytokines IFN-γ and IL-17 in the colonic mucosa of the infected and treated mice (see Fig. A and B, Supplemental Digital Content 1, http://links.lww.com/IBD/A714). An additional readout of colitis pathology known to correlate well with the histopathological gold standard is colon length. Mice in the positive control group exhibited substantially shorter colons than negative controls that had never been exposed to DSS; *H. pylori* infection or extract treatment restored normal colon length (Fig. 1C). Finally, high resolution endoscopy[22] was used to quantitatively assess colitis in a subset of the mice shown in Figure 1A. Whereas DSS-treated mice of the positive control group were characterized by thickening and intransparency of the colon, mucosal bleeding, abundant fibrin, granularity of the mucosal surface, and loose stools, all of these parameters were less severe in the *H. pylori*–infected or extract-treated mice (Fig. 1D, E). Consequently, the overall colonoscopy scores assigned on a scale from 0 to 15[22] for

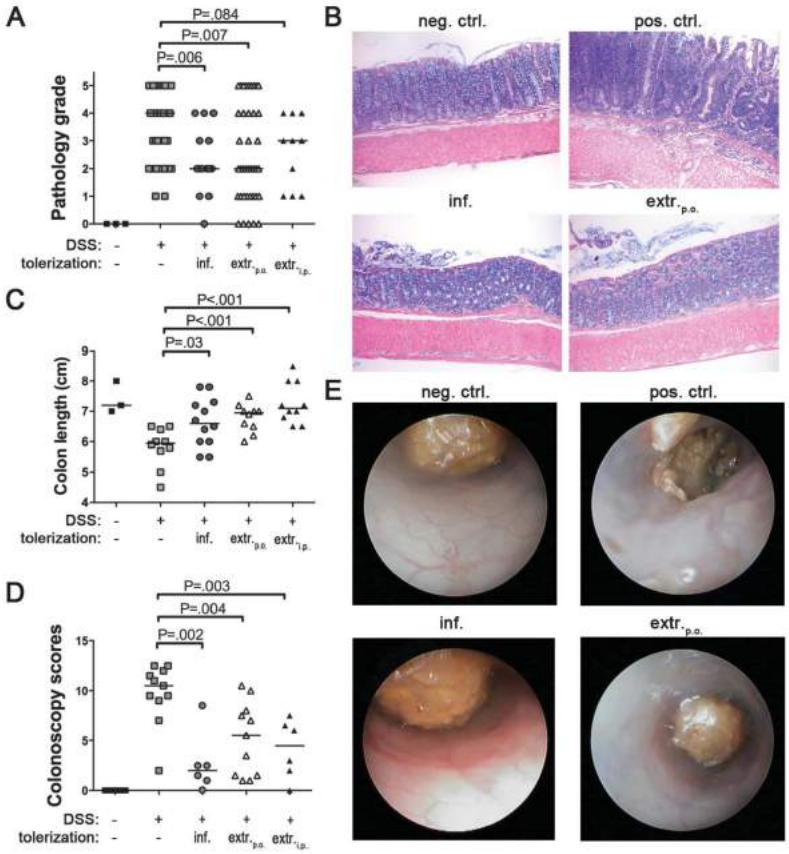

FIGURE 1. *Helicobacter pylori* infection and *H. pylori* extract treatment protect against DSS-induced colitis. C57BL/6 mice were either infected with 1 orogastric dose of live *H. pylori* (strain PMSS1) at 7 days of age or received 3 weekly doses of 200 μg *H. pylori* extract from the day of the first DSS cycle onwards, administered either i.p. or perorally (p.o.) and were subjected to three 5-day cycles of 2% DSS in the drinking water. A and B, Histopathology scores and representative micrographs of Giemsa-stained tissue sections. Pooled data from 2 (inf., extr.$_{i.p.}$) to 5 (pos. ctrl., extr.$_{p.o.}$) independent studies are shown. C, Colon lengths as determined from cecum to distal rectum for a subset of the mice shown in (A). D and E, Colonoscopy scores and representative endoscopic images of a subset of the mice shown in (A). A, C, and D, Each data point represents 1 mouse; horizontal lines indicate the medians. inf., infected; extr., extract-treated; pos. ctrl., positive control; neg. ctrl., negative control.

the above-mentioned parameters were reduced from an average of 10 (positive controls) to 5 (infected and extract treated; Fig. 1D). Interestingly, lower doses of extract (<10 mg/kg body weight) or once-weekly treatment with the 10 mg/kg dose were not sufficient to confer protection (data not shown), whereas the i.p. injection of *H. pylori* extract conferred a level of protection that was quite similar to the protection provided by orogastric delivery (Fig. 1A–E).

To determine whether the observed protective effects are specific to the DSS model of barrier disruption followed by microbiota-induced autoinflammation, we assessed the efficacy of extract treatment in the T-cell transfer-mediated model of colitis.[26] Lymphopenic recipients of adoptively transferred naive $CD4^+$ T cells were either subjected to 3 times weekly treatment with *H. pylori* extract or remained untreated. Whereas the recipients of naive T cells in the positive control group developed severe

colitis as determined by loss of body weight as well as histopathological and endoscopical analysis, all symptoms were strongly reduced in extract-treated animals (see Fig. C–E, Supplemental Digital Content 1, http://links.lww.com/IBD/A714). All measured parameters are thus consistent with protective effects of live *H. pylori* infection and/or extract treatment on the development of chronic colitis in 2 models of IBD. No adverse effects of the extract treatment could be detected on the gastric mucosa of a non-colitic control group (see Fig. F–G, Supplemental Digital Content 1, http://links.lww.com/IBD/A714), neither in the presence or absence of an experimental *H. pylori* infection, indicating that the treatment does not induce or aggravate *H. pylori*–associated gastric pathology.

Helicobacter pylori Infection or Extract Treatment Do not Ameliorate Experimental Autoimmune Encephalomyelitis or Type 1 Diabetes

Hypothesizing that other autoinflammatory or autoimmune conditions might also be prevented or ameliorated by *H. pylori*, we examined the effects of *H. pylori* infection or extract treatment in models of multiple sclerosis and T1D. To assess the effects of *H. pylori* on multiple sclerosis–like autoimmune neuroinflammation, we immunized wild-type C57BL/6 mice with MOG_{35-55} peptide to trigger EAE. All mice developed central nervous system inflammation as determined by scoring of the progressive paralysis of tail and hind limbs within ~9 to 13 days postimmunization; *H. pylori* infection or 3 times weekly treatment with *H. pylori* extract did not measurably affect the course of disease (Fig. 2A, B); if anything, extract treatment made the condition worse. Similarly, *H. pylori* infection of female NOD mice, which spontaneously develop pancreatic islet inflammation and represent a widely used model of T1D, did not result in improved pancreatic histopathology (Fig. 2C, D). Finally, in a parallel model of inducible T1D, male NOD mice were subjected to a single dose of cyclophosphamide to trigger pancreatic islet inflammation; as in the spontaneous model, neither live infection nor extract treatment alleviated disease symptoms (Fig. 2E), suggesting that T1D develops regardless of the presence of *H. pylori*. We verified that *H. pylori* is fully competent to colonize NOD mice, albeit at lower levels than the C57BL/6 mice used in the DSS-induced colitis model (Fig. 2F). In conclusion, our results indicate that prototypical autoimmune diseases are not controlled by live *H. pylori* and therefore also fail to respond to *H. pylori* extract treatment.

Protection Against Colitis Conferred by Live *H. pylori* or Its Extract Is Accompanied by MUC2 Upregulation and Depends on the NLRP3 Inflammasome and IL-18 Signaling

The most striking endoscopic feature of mice that are protected against experimentally induced colitis by either live *H. pylori* infection or extract treatment is their abundant mucus production (Fig. 1E). The predominant intestinal mucin, MUC2, is produced by goblet cells and forms an insoluble barrier that protects the intestinal epithelium against colonization by gut microbes.[27] We investigated the expression of MUC2 transcript and, in accordance with the endoscopy results, found it to be strongly upregulated in the intestinal mucosa of *H. pylori*–infected and extract-treated mice relative to colitic controls (Fig. 3A). The intestinal transcription factor CDX2, which regulates MUC2 production,[28] is upregulated in a similar manner in the protected mice (Fig. 3B). Having identified a copy of the SMAD binding motif CAGACA[29] in the CDX2 promoter sequence, we speculated that the regulatory cytokine TGF-β, known to be critically involved in *H. pylori*–specific immunomodulation and in intestinal homeostasis,[20,30] might play a role in *H. pylori*–induced colitis protection. The expression pattern of TGF-β indeed paralleled those of MUC2 and CDX2 (Fig. 3C), suggesting that the TGF-β-CDX2-MUC2 axis represents a strong correlation of, or is functionally involved in, colitis protection. To examine whether MUC2 induction by *H. pylori* extract is a phenomenon linked to DSS treatment, we treated a large group of mice with 3 times weekly doses of *H. pylori* extract without exposing them to DSS at any time; this treatment recapitulated the strong MUC2 induction seen in DSS-treated mice (Fig. 3D), indicating that intestinal MUC2 upregulation is a direct result of the exposure to *H. pylori* extract rather than a consequence of the protection from DSS colitis.

We and others have reported recently that *H. pylori* activates the inflammasome and caspase-1 to induce the processing and secretion of IL-1β and IL-18.[31,32] IL-18 signaling is required for the protection against allergic asthma that is conferred by live infection[17] and *H. pylori* extract treatment.[18] Several polymorphisms in the IL-18 gene are known to contribute to UC susceptibility.[33] To test whether a functional NLRP3 inflammasome, the predominant inflammasome detecting *H. pylori*,[32] and IL-18 signaling proficiency are required for *H. pylori* extract-mediated protection, we subjected mice lacking NLRP3, IL-18, IL-18 receptor, or the adaptor protein MyD88 to DSS-induced colitis and treated the mice with *H. pylori* extract. None of these strains were protected against DSS-induced colitis, despite the fact that wild-type mice examined in the same experiment exhibited a typical level of protection (Fig. 3E). The activation of the NLRP3 inflammasome and subsequent secretion of IL-18 upon extract treatment thus are important prerequisites of *H. pylori* extract–mediated colitis protection.

DISCUSSION

The combined results presented here demonstrate that *H. pylori*, administered either live or in extract form, confers protection against IBD in standard mouse models of the disease. Our data are consistent with a strong inverse correlation of *H. pylori* seropositivity with the risk of developing both CD and UC,[13,15] as well as with experimental data documenting a protective effect of live *H. pylori* infection on acute *Salmonella typhimurium*–induced

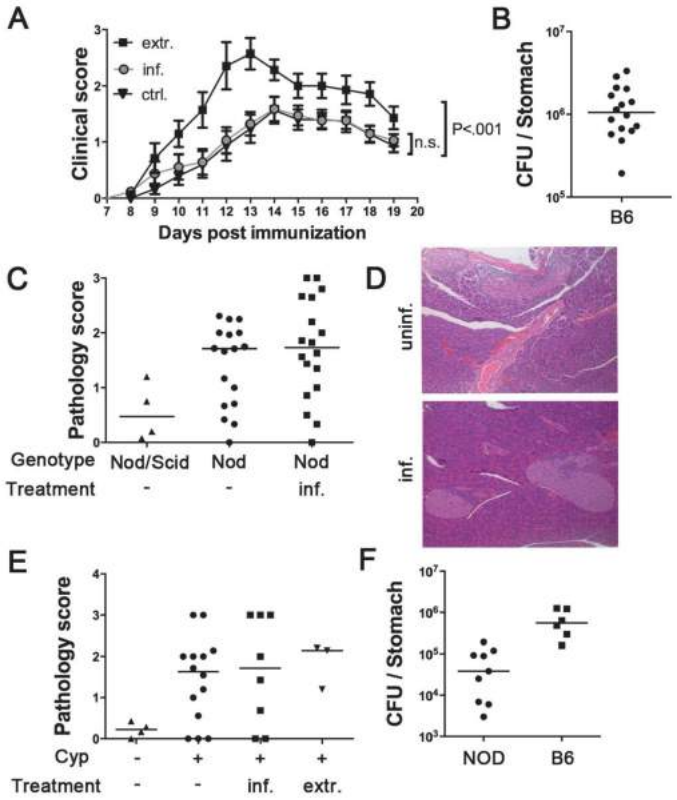

FIGURE 2. *Helicobacter pylori* infection and *H. pylori* extract treatment fail to protect against EAE and T1D. A and B, C57BL/6 mice were either infected on day 7 of age with *H. pylori* (16 mice) or received 3 weekly i.p. doses of 200 μg *H. pylori* extract from day 7 of age onwards (12 mice), or remained untreated (24 mice). All mice were immunized with MOG_{35-55} peptide at 6 weeks of age and monitored for EAE symptoms for 19 days thereafter. Averages ± SDs of EAE scores of the 3 treatment groups are shown in (A). Colony-forming units per stomach as determined by plating and colony counting are shown in (B) for all infected mice. Pooled data from 2 independent studies are shown in (A) and (B). C and D, Female NOD mice were either infected on day 7 of age with *H. pylori* (18 animals) or remained uninfected (17 animals) and were killed at 20 weeks of age for the histopathological assessment of spontaneously occurring insulitis. Four nondiabetic NOD/SCID mice were included as negative controls. Histopathology scores are shown in (C) along with representative micrographs of the NOD groups in (D). E and F, Male NOD mice were either infected on day 7 of age with *H. pylori* (8 mice) or received 3 weekly i.p. doses of 200 μg *H. pylori* extract from day 7 of age onwards (3 mice) or remained untreated (14 mice). All mice except for 4 negative controls received 1 i.p. dose of 5 mg cyclophosphamide at 10 weeks of age and were assessed histopathologically at 20 weeks of age. Histopathology scores and colony-forming units, relative to age-matched C57BL/6 mice, are shown in (E) and (F). inf., infected; extr., extract-treated; pos. ctrl., positive control; neg. ctrl., negative control.

typhlocolitis.[34] The enteroprotective effects of *H. pylori* in the latter model have been attributed to immunoregulatory sequences in the genomic DNA of *H. pylori*, which downregulate proinflammatory cytokine production and induce tolerogenic properties in dendritic cells.[35] Here, we find that the *H. pylori*–specific activation of the NLRP3 inflammasome and the subsequent processing and secretion of mature IL-18 are critical events in colitis protection. We and others have shown earlier that *H. pylori* efficiently activates the (NLRP3) inflammasome in innate immune cells to induce the autoproteolytic cleavage of caspase-1 and

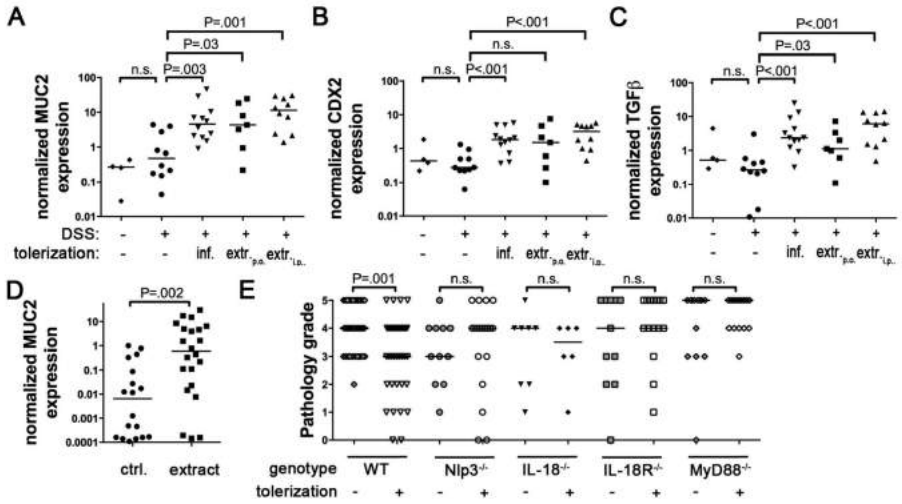

FIGURE 3. *Helicobacter pylori* infection and extract treatment induces MUC2 expression and confers protection against DSS colitis in a NLRP3-dependent and IL-18–dependent manner. A–C, C57BL/6 mice were treated as described in Figure 1. Colonic mucosal tissue was subjected to RNA extraction and qRT-PCR for MUC2, CDX2, and TGF-β transcripts; expression levels were normalized to GAPDH. D, 6-week-old mice that had been treated 3 times weekly with 200 μg *H. pylori* extract from day 7 of age onwards were subjected to MUC2 qRT-PCR, normalized to GAPDH. No DSS was administered to these mice. E, Mice of the indicated genotypes were treated with *H. pylori* extract as described in Figure 1 and exposed to 3 cycles of DSS in the drinking water. Pooled histopathology scores of 3 independent studies are shown.

the processing of pro-IL-1β and pro-IL-18 into the mature cytokines.[31,32,36] Interestingly, the 2 caspase-1 substrates fulfill very different, even opposing, functions in the *H. pylori*/host interaction. Whereas IL-1β has a strong pro-inflammatory role, promoting the differentiation of *H. pylori*–specific Th1 and Th17 cells and immune control of *H. pylori* on the one hand and infection-associated gastric immunopathology on the other,[31,32] the net effect of IL-18 signaling in the context of *H. pylori* infections is anti-inflammatory rather than pro-inflammatory. Expression of IL-18 and signaling through its receptor is dispensable for *H. pylori* infection control; in fact, mice that lack either the ligand or the receptor are capable of controlling *H. pylori* loads more efficiently because of their unrestricted *H. pylori*–specific Th17 responses.[17,31] As another consequence of excessive gastric Th17 activation, IL-18$^{-/-}$ and IL-18R$^{-/-}$ mice develop severe infection-associated immunopathology.[31] *Helicobacter pylori*–induced IL-18 is not only required for the prevention of immunopathology and for the protection against IBDs as shown here but has also emerged as a critical factor in *H. pylori*–induced asthma prevention.[17,18] In asthma, IL-18, which is secreted in large amounts by DCs that have been exposed to *H. pylori* in vitro or in vivo, promotes the differentiation of naive T cells into FoxP3^{+}CD25^{+} regulatory T cells with highly suppressive activity.[17] CD4^{+}CD25^{+} T cells isolated from the mesenteric lymph nodes of wild type, but not IL-18$^{-/-}$ and IL-18R$^{-/-}$ donors, confer asthma protection to naive recipients.[17] Whether IL-18–mediated protection in the colitis model depends similarly on the induction and function of Tregs remains to be determined. In the gut, an alternative IL-18–dependent scenario can be envisioned that involves the production of large amounts of intestinal mucins, which are known to protect against colitis.[37] Our observation that MUC2 mucus production is induced by live *H. pylori* and extract, and correlates well with protection, is reminiscent of a recently described innate immune mechanism linking the microbiota-induced activation of the NLRP6 inflammasome to goblet cell mucus hypersecretion.[37] Whereas NLRP6-dependent mucus production was attributed to mucin granule exocytosis[37] rather than transcriptional activation of MUC2 gene expression as demonstrated here, the net effect of enhanced protective mucus production is comparable in both scenarios. In conclusion, we propose here that the activation of mucus production through the NLRP3/caspase-1/IL-18 axis by *H. pylori* extract or live bacteria forms the mechanistic basis for a possible new treatment modality for IBD that is projected to be safe and cost-effective and exploits the immunomodulatory properties of a naturally occurring infectious agent known to be inversely associated with IBD risk in human populations.

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10.2 *Helicobacter* urease-induced activation of the TLR2/NLRP3/IL-18 axis protects against asthma

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Contribution: I helped perform experiments for figures 3 and S7.

The Journal of Clinical Investigation BRIEF REPORT

Helicobacter urease–induced activation of the TLR2/NLRP3/IL-18 axis protects against asthma

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Inflammasome activation and caspase-1–dependent (CASP1-dependent) processing and secretion of IL-1β and IL-18 are critical events at the interface of the bacterial pathogen *Helicobacter pylori* with its host. Whereas IL-1β promotes Th1 and Th17 responses and gastric immunopathology, IL-18 is required for Treg differentiation, *H. pylori* persistence, and protection against allergic asthma, which is a hallmark of *H. pylori*–infected mice and humans. Here, we show that inflammasome activation in DCs requires the cytoplasmic sensor NLRP3 as well as induction of TLR2 signaling by *H. pylori*. Screening of an *H. pylori* transposon mutant library revealed that pro–IL-1β expression is induced by LPS from *H. pylori*, while the urease B subunit (UreB) is required for NLRP3 inflammasome licensing. UreB activates the TLR2-dependent expression of NLRP3, which represents a rate-limiting step in NLRP3 inflammasome assembly. *ureB*-deficient *H. pylori* mutants were defective for CASP1 activation in murine bone marrow–derived DCs, splenic DCs, and human blood-derived DCs. Despite colonizing the murine stomach, *ureB* mutants failed to induce IL-1β and IL-18 secretion and to promote Treg responses. Unlike WT *H. pylori*, *ureB* mutants were incapable of conferring protection against allergen-induced asthma in murine models. Together, these results indicate that the TLR2/NLRP3/CASP1/IL-18 axis is critical to *H. pylori*–specific immune regulation.

Introduction

Persistent infection of the gastric mucosa with *H. pylori* causes gastritis (1) and represents a major risk factor for the development of gastric cancer (2) but has also been inversely linked to the risk of allergic and chronic inflammatory diseases (3, 4). The outcome of the *H. pylori*/host interaction is determined both by host and bacterial genetic factors (5) as well as the infected individual's predominant T cell response to *H. pylori*: whereas asymptomatic carriers generate *H. pylori*–specific Tregs, patients with peptic ulcer are characterized by Th1/Th2-biased, pathogenic T effector responses (6). Treg-predominant responses are particularly pronounced in children (7) and can be recapitulated in experimental models of neonatal *H. pylori* infection (8), in which they are required for protection against allergen-induced asthma (9). *H. pylori* activates caspase-1 (CASP1) in infected macrophages and DCs and induces the processing and secretion of the CASP1-dependent cytokines IL-1β and IL-18 by triggering the assembly and activation of an ASC- and NLRP3-containing inflammasome (10–12). CASP1 activation by *H. pylori* has both proinflammatory and anti-inflammatory consequences that are differentially mediated by its cytokine substrates (10). IL-1β exerts proinflammatory effects that promote Th1- and Th17-driven *H. pylori* control and gastric immunopathology (10, 11); in contrast, IL-18 signaling restricts severe gastric immunopathology and contributes to asthma protection by promoting Treg differentiation (10, 13). Here, we show using in vitro and in vivo infection models that NLRP3 inflammasome activation by *H. pylori* requires licensing through TLR2. A saturating transposon (tn) library screen revealed a critical and previously unrecognized role for *H. pylori*'s urease enzyme in promoting the TLR2-dependent transcriptional activation of NLRP3 expression, CASP1 activation, and cytokine processing as well as Treg differentiation and asthma protection.

Conflict of interest: The authors have declared that no conflict of interest exists.
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Results and Discussion

H. pylori activates CASP1 and induces IL-1β secretion by DCs in an ASC-, NLRP3-, and TLR2-dependent manner. Having shown previously that *H. pylori* exposure activates CASP1 and induces the secretion of mature IL-1β and IL-18 in bone marrow–derived DCs (BMDCs) (10), we sought to identify host determinants of inflammasome activation by *H. pylori*. BMDCs from WT, *Nlrc4*$^{-/-}$, *Nlrp6*$^{-/-}$, *Nlrp3*$^{-/-}$, *Aim2*$^{-/-}$, and *Asc*$^{-/-}$ mice, and from mice lacking various TLRs and adaptor molecules, were cocultured with two different strains of *H. pylori* and examined with respect to CASP1 activation and IL-1β secretion. Whereas the inflammasome sensors NLRC4, NLRP6, and AIM2 were dispensable for CASP1 activation and IL-1β secretion, both processes were found to be dependent on the bipartite adaptor protein ASC as well as the cytoplasmic inflammasome sensor NLRP3 (Figure 1, A–D, and Supplemental Figure 1, A–E; supplemental material available online with this article; doi:10.1172/JCI79337DS1). Surprisingly, we found that CASP1 activation and IL-1β secretion, but not the transcriptional activation of IL-1β, required surface-exposed TLR2 (Figure 1, E–G). Other surface and endosomal TLRs known to contribute to innate immune recognition of Gram-negative pathogens, i.e., TLR4, TLR5, and TLR9 or the IL-1 receptor, were not required for IL-1β secretion (Figure 1, E–G, and Supplemental Figure 2, A–I). Similarly, we could not

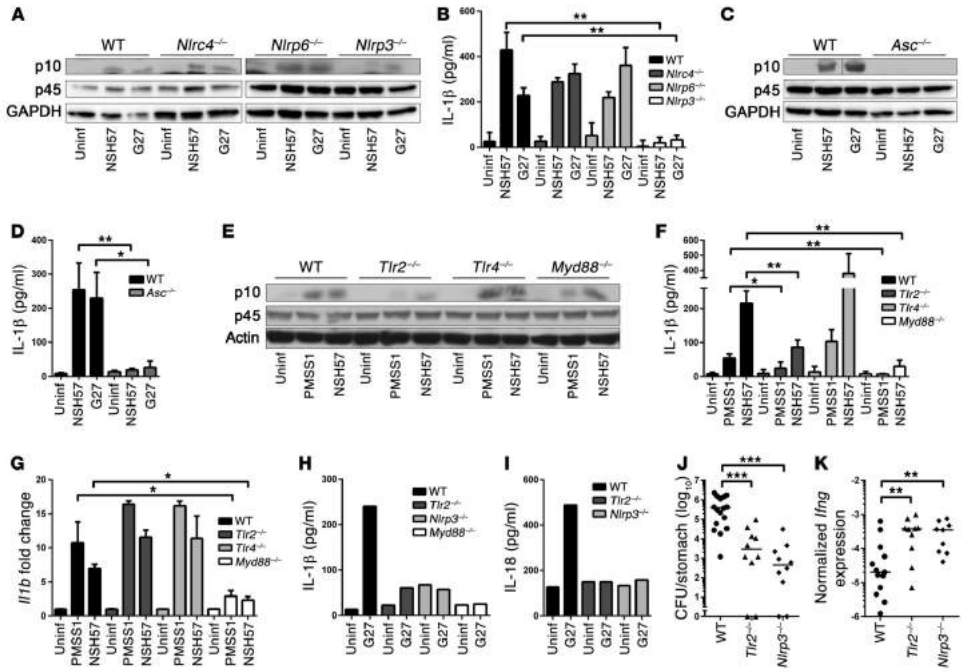

Figure 1. CASP1 activation by *H. pylori* depends on NLRP3, ASC, and TLR2. (**A**–**G**) BMDCs from mice of the indicated genotypes were infected overnight with *H. pylori* NSH57, G27, and/or PMSS1. (**A**, **C**, and **E**) Western blot analysis of CASP1 activation (p10) in the cell supernatant compared to full-length CASP1 p45 and GAPDH in the extract. Representative results of 3 independent experiments are shown ($n = 3$). (**B**, **D**, and **F**) IL-1β ELISA of culture supernatants; cells were prestimulated with *E. coli* LPS prior to infection. Uninf, uninfected. (**G**) *Il1b* transcription, as measured by qRT-PCR (normalized to *Gapdh* and to uninfected controls). Mean + SD of 3 independent experiments is shown ($n = 3$). (**H** and **I**) CD11c$^+$ splenic DCs were infected overnight with G27. (**H**) IL-1β and (**I**) IL-18 secretion was measured by ELISA. Representative data of 3 independent experiments are shown ($n = 3$). (**J** and **K**) Mice were infected for 1 month with PMSS1 prior to the quantification of (**J**) gastric colonization and (**K**) *Ifng* expression. Pooled data from 2 studies are shown ($n = 2$). Horizontal lines indicate medians. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, Mann-Whitney *U* test. Error bars represent mean + SD.

detect a contribution of the Nod-like receptor NOD2 or of the ATP sensor P2X7R to these processes (Supplemental Figure 2, J–L). Of two known adaptor molecules relaying signals downstream of the TLRs, only MyD88, but not TRIF, was involved in IL-1β expression and secretion (Figure 1, E–G, and Supplemental Figure 2, M and N). A recently identified noncanonical inflammasome activation pathway involving TRIF- and IRF3/7-mediated type I IFN production and signaling (14) was dispensable for *H. pylori*-induced inflammasome activation (Supplemental Figure 2, N–R). The critical role of TLR2, MyD88, and NLRP3 in *H. pylori*–induced IL-1β secretion was confirmed with immunomagnetically isolated CD11c$^+$ splenic DCs (Figure 1H and Supplemental Figure 2S). IL-18 secretion by splenic DCs also required TLR2 and NLRP3 (Figure 1I).

CASP1$^{-/-}$ mice, and mice lacking either IL-18 or its receptor, control *Helicobacter* infections more effectively than WT animals, because they fail to peripherally induce Tregs (10, 13). Experimental infection of *Nlrp3*$^{-/-}$ and *Tlr2*$^{-/-}$ mice with the *H. pylori* isolate PMSS1 recapitulates this phenotype: both strains were colonized at lower levels and exhibited higher gastric mucosal IFN-γ expression (Figure 1, J and K), which correlates well with higher frequencies of IFN-γ–expressing CD4$^+$ T cells in the mesenteric lymph nodes (MLNs) of *Tlr2*$^{-/-}$ mice relative to those in WT mice (Supplemental Figure 2T). The combined results suggest that *H. pylori* activates the inflammasome in a TLR2- and NLRP3-dependent manner and benefits from this process because it promotes *H. pylori* persistence.

Genome-wide screening for factors involved in IL-1β secretion reveals a role for H. pylori LPS and urease. Known activators of the NLRP3 inflammasome include both foreign and endogenous compounds, with the best-understood being urate crystals, asbestos, ATP, and bacterial pore-forming toxins (15). We were able to exclude a role for the *H. pylori* immunomodulator γ-glutamyltranspeptidase GGT and the Cag pathogenicity island in CASP1 activation and IL-1β secretion (Supplemental Figure 3, A and B). To search for *H. pylori* factors involved in inflammasome activation in a genome-wide manner, we took advantage of a previously described tn mutant library (16). As IL-1β secretion by *H. pylori*–

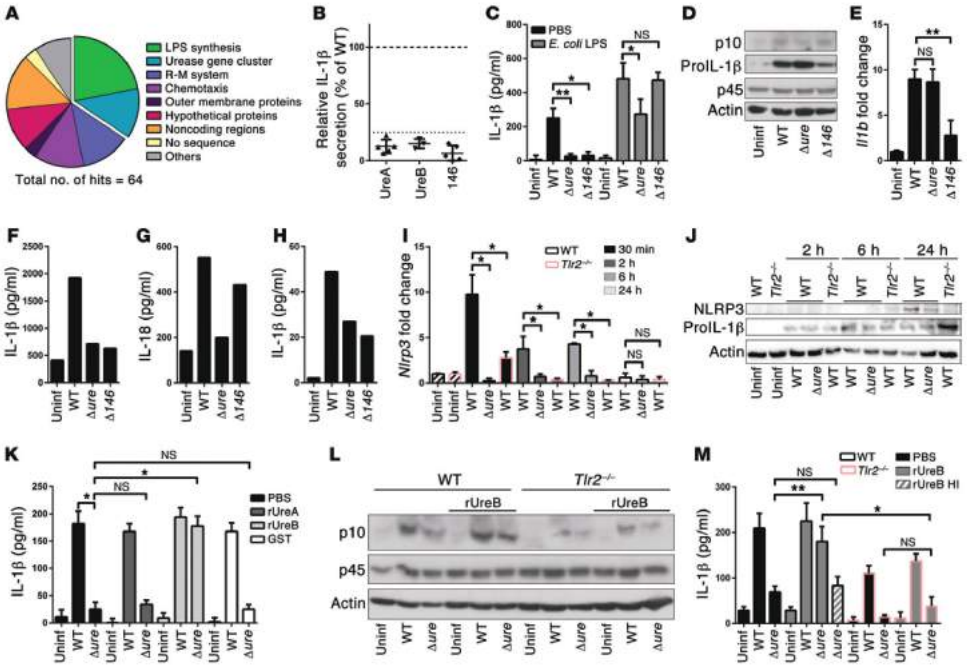

Figure 2. IL-1β secretion upon *H. pylori* infection requires LPS-induced transcriptional activation of pro-IL-1β and urease- and TLR2-dependent expression of NLRP3. (**A**) Genome-wide screening for *H. pylori* tn mutants incapable of IL-1β secretion identifies the indicated mutant categories. R-M, restriction modification. (**B**) Relative IL-1β secretion of individual tn clones with insertions in *ureA*, *ureB*, and HPG27_146. (**C**–**E** and **I**–**M**) Murine BMDCs, (**F** and **G**) splenic CD11c$^+$ DCs, and (**H**) human blood-derived DCs were infected overnight or for the indicated time points with G27 WT, Δ*ure*, or Δ*146* strains of *H. pylori* and analyzed by (**C**, **F**, **H**, **K**, and **M**) IL-1β ELISA; (**G**) IL-18 ELISA; (**D**, **J**, and **L**) CASP1 p10 and p45, pro–IL-1β, and NLRP3 Western blotting; and (**E** and **I**) qRT-PCR (normalized to *Gapdh* and to uninfected controls). BMDCs were prestimulated with *E. coli* LPS where indicated, and 1 μg/ml recombinant GST-tagged UreA, UreB (native or heat-inactivated [HI] for 10 minutes at 70°C), or GST was added to cocultures as noted. Pooled data from 3 to 4 independent experiments are show in **C**, **E**, **I**, **K**, and **M**; a representative of 2 experiments is shown in **F**–**H**. $^*P \le 0.05$, $^{**}P \le 0.01$, Mann-Whitney *U* test. Error bars represent mean + SD.

exposed DCs is dependent on CASP1 (10), we opted for IL-1β ELISA as a screening readout (Supplemental Figure 3C). The insertion sites of 64 mutants with defects in inducing IL-1β secretion (<25% of the corresponding WT infection) were sequenced and mapped to 32 different loci (Supplemental Table 1). Loci belonging to two mutant categories were identified repeatedly; these harbored tn insertions in genes involved in LPS biosynthesis and in the urease gene cluster (Figure 2, A and B). The LPS synthesis gene hit most often was LPS-1,2-glycosyltransferase (HPG27_146). LPS from *H. pylori* deficient for this gene lack the O-side chain and therefore Lewis antigens (Supplemental Figure 4A). A gene-specific deletion mutant (Δ*146*) in strain G27 recapitulated the tn mutant phenotype, as it failed to induce IL-1β secretion in BMDCs (Figure 2C). The phenotype of this mutant was attributable to its failure to induce pro–IL-1β expression at the transcriptional level, rather than to a defect in CASP1 activation, and could be rescued by *E. coli* or *H. pylori* LPS (Figure 2, C–E, and Supplemental Figure 4, B and C). IL-1β expression upon stimulation with both types of LPS was MyD88- and TLR4-dependent (Supplemental Figure 4D).

Remarkably, of the 8 tn insertions mapping to the urease gene cluster, all affected 2 genes encoding the structural urease subunits, *ureA* or *ureB* (Supplemental Table 1). A gene-specific deletion mutant lacking both UreA and UreB proteins (G27Δ*ure*, Supplemental Figure 5A) phenocopied the effect of the tn insertion mutants, which could be attributed to its failure to activate CASP1 (Figure 2, C and D). In contrast, *Il1b* transcription was normal (Figure 2E). Coculturing of murine splenic CD11c$^+$ DCs and human blood-derived DCs confirmed the defect of the *H. pylori* Δ*ure* and Δ*146* mutants with respect to IL-1β secretion; in contrast, the secretion of IL-18, which does not require transcriptional activation, was almost at WT levels in the case of the Δ*146* mutant (Figure 2, F–H). In summary, our screen identified *H. pylori* factors regulating CASP1-dependent cytokine secretion at two distinct levels, one transcriptional and one posttranslational.

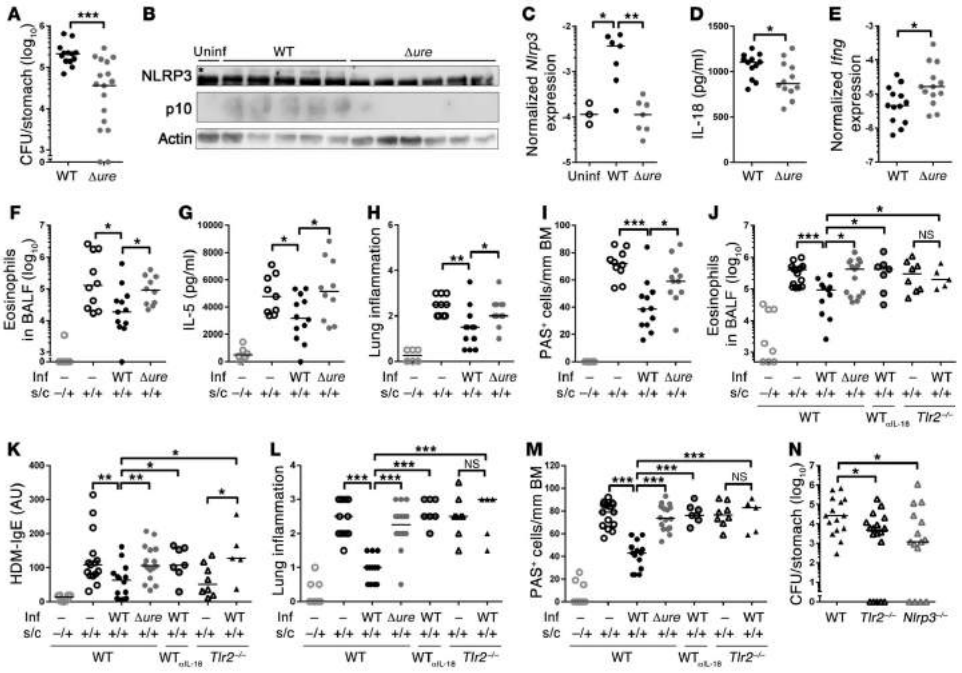

Figure 3. *H. pylori* urease is required for CASP1 activation, persistence, and asthma protection in neonatally infected mice. (**A**–**E**) Neonatal C57BL/6 mice were infected for 1 month with WT or Δ*ure H. pylori* PMSS1 and assessed with respect to (**A**) gastric colonization, (**B**) gastric mucosal NLRP3 and CASP1 (asterisk indicates the NLRP3-specific band), (**C**) *Nlrp3* expression, (**D**) IL-18, and (**E**) *Ifng* expression, as analyzed by (**B**) Western blotting, (**D**) ELISA, and/or (**C** and **E**) qRT-PCR. (**F**–**M**) Neonatally infected mice were additionally sensitized and challenged (s.c.) with (**F**–**I**) ovalbumin or (**J**–**M**) house dust mite (HDM) allergen starting at 4 weeks after infection to induce allergic asthma; αIL-18 mAb was administered weekly starting at the time of infection. Inf, infection. (**F** and **J**) Eosinophils in 1 ml of bronchoalveolar lavage fluid (BALF). (**G**) IL-5 ELISA of ovalbumin-restimulated lung single cell preparations. (**H** and **L**) Lung inflammation, as assessed on H&E-stained sections. (**I** and **M**) Goblet cell metaplasia, as quantified on PAS-stained sections. BM, basement membrane. (**K**) House dust mite–specific serum IgE, as determined by ELISA. (**N**) *H. pylori* colonization of WT, *Tlr2*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice 1 month after infection. Symbols represent individual animals, and horizontal lines indicate the medians. Pooled data from 2 (**A**–**E** and **N**) and 3 (**F**–**M**) independent experiments are shown. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, Mann-Whitney *U* test.

Given the similarities in outcome of TLR2 deficiency of the host on the one hand and urease deficiency of the bacteria on the other (i.e., lack of CASP1 activation), we hypothesized that *H. pylori* urease might provide a TLR2-mediated signal to promote inflammasome/CASP1 activation. As NLRP3 expression can be primed by TLR signaling, we asked whether NLRP3 transcript and protein levels in BMDCs are affected by *H. pylori* exposure. Indeed, WT *H. pylori* infection efficiently induced NLRP3, but not AIM2 or NLRC4, expression at the transcript and protein levels in a TLR2-, MyD88-, and NF-κB–dependent manner; this was not observed in BMDCs infected with Δ*ure H. pylori* (Figure 2, I and J; Supplemental Figure 5, B–H; and data not shown). Interestingly, the defect of the Δ*ure* mutant with respect to CASP1 activation and IL-1β secretion could be rescued by recombinant UreB, but not UreA or the GST tag control; this effect was only seen in WT, but not in *Tlr2*$^{-/-}$, BMDCs (Figure 2, K–M). Heat-inactivated UreB had no effect on the two processes (Figure 2M and data not shown). Taken together, the results suggest that UreB signals via TLR2 to prime NLRP3 expression, which appears to be a rate-limiting step in *H. pylori*–induced inflammasome activation and IL-1β processing. This is particularly interesting because TLR2 has not yet been described to be activated by bacterial (non-lipo-) proteins.

H. pylori urease is required for CASP1 activation, Treg responses, and asthma protection in vivo. To investigate the consequences of LPS and urease deficiency in vivo, Δ*ure* and Δ*146* mutants were generated in the mouse-colonizing strains PMSS1 and/or SS1 and used for experimental infections of adult or neonatal C57BL/6 mice. Whereas Δ*146* failed to colonize under all circumstances (data not shown), the PMSS1Δ*ure* mutant colonized adult infected mice at WT levels for at least 3 months, without evidence of having regained urease expression (Supplemental Figure 6, A and B). In contrast, SS1Δ*ure* consistently failed to colonize (data

not shown), confirming that urease proficiency is required for mouse colonization in certain strain backgrounds (17). Interestingly, PMSS1Δ*ure* induced significantly less gastric production of IL-1β and IL-18 and less active CASP1 than the parental WT strain (Supplemental Figure 6, C–E). In line with the increased gastric IFN-γ expression of *Tlr2*$^{-/-}$ and *Nlrp3*$^{-/-}$ animals (Figure 1K), Δ*ure*-infected mice exhibited higher gastric mucosal IFN-γ expression and more IFN-γ^{+}CD4^{+} cells in the MLNs than infected WT animals (Supplemental Figure 6, F and G). A similar pattern was observed in neonatally infected animals, in which CASP1 activation, NLRP3 expression, and IL-18 secretion were also found to depend on urease proficiency of *H. pylori*; moreover, colonization levels of the Δ*ure* mutant were strongly reduced relative to the WT strain in neonatally infected mice (Figure 3, A–D). As in adult infected mice, the Δ*ure* mutant elicited higher *Ifng* expression (Figure 3E). Interestingly, *H. pylori* urease was further required for the efficient protection against allergen-induced asthma that is a hallmark of neonatally infected mice. All examined parameters of ovalbumin-induced allergic asthma, i.e., bronchoalveolar eosinophilia, lung inflammation, and goblet cell metaplasia as well as pulmonary Th2 cytokine production, were clearly reduced in infected WT animals but not in Δ*ure*-infected animals (Figure 3, F–I, and Supplemental Figure 7A). Similar results were obtained in the house dust mite model of allergic asthma (Figure 3, J–M). Moreover, protection was abrogated by a blocking antibody targeting IL-18 and in *Tlr2*$^{-/-}$ mice (Figure 3, J–M), which, similar to *Nlrp3*$^{-/-}$ mice, had a lower bacterial burden than WT mice (Figure 3N). Protection against ovalbumin-induced asthma could be adoptively transferred via immunomagnetically purified CD25^{+} Tregs from *H. pylori* infected WT donors but not Δ*ure*-infected donors; Tregs from *H. pylori* infected WT *Tlr2*$^{-/-}$ and *Nlrp3*$^{-/-}$ animals also failed to confer protection (Supplemental Figure 7, B and C). The quantification of CD25^{+}FoxP3^{+} Tregs in MLNs further revealed lower Treg frequencies in Δ*ure*-infected mice relative to infected WT mice and *Tlr2*$^{-/-}$ mice relative to WT mice (Supplemental Figure 7, D and E). In summary, the findings described here document a previously unrecognized role of *H. pylori* urease in innate immune recognition and *H. pylori* persistence that presumably is unrelated to its function in acid resistance. Here, we show that UreB promotes the TLR2-dependent expression of NLRP3, a critical component of the inflammasome that is required for CASP1 activation and IL-1β/IL-18 processing (see model in Supplemental Figure 8). Infection with urease gene deletion mutants phenocopies the effects of TLR2 and NLRP3 deficiency. The combined results confirm a critical contribution of the TLR2/NLRP3/CASP1/IL-18 axis to microbially induced immune regulation and introduce the *H. pylori* urease as a novel immunomodulator of this important human pathobiont.

Methods

Animal experimentation. C57BL/6 WT, *Casp1*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr5*$^{-/-}$, *Tlr9*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*$^{-/-}$, *Nlrp3*$^{-/-}$, *Ifnar*$^{-/-}$, *Irf7*$^{-/-}$, *Nod2*$^{-/-}$, *P2rx7*$^{-/-}$, and *Aim2*$^{-/-}$ mice were originally obtained from Charles River Laboratories. *Nlrc4*$^{-/-}$ and *Asc*$^{-/-}$ mice were provided by Genentech. *Nlrp6*$^{-/-}$ mice were provided by Millenium Pharmaceuticals. *Il1r*$^{-/-}$ mice were provided by Manfred Kopf. Mice were infected orally with 10^{8} CFU *H. pylori* PMSS1 at 6 weeks or 7 days of age. Bacterial colonization was assessed by colony counting. The procedures used for asthma induction and cytokine quantification by qPCR, ELISA, and FACS are described in the Supplemental Methods.

H. pylori strains, infection of DCs, and tn library screening. H. pylori strains and culture conditions as well as the procedures used for the differentiation and immunomagnetic isolation of DCs and for tn library screening are described in the Supplemental Methods, along with protocols for Western blotting and purification of recombinant proteins.

Statistics. GraphPad Prism (GraphPad Software) was used for statistical analyses. All *P* values were calculated by Mann-Whitney *U* test.

Study approval. All animal experimentation was reviewed and approved by the Veterinary Office of the canton of Zurich (Zurich, Switzerland) (licenses 24/2013 and 170/2014 to A. Müller).

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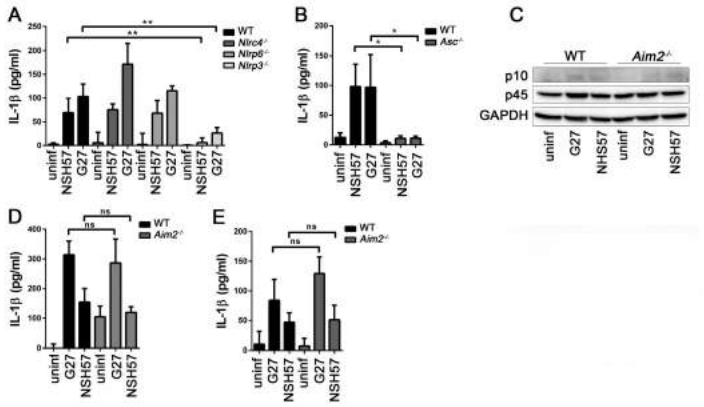
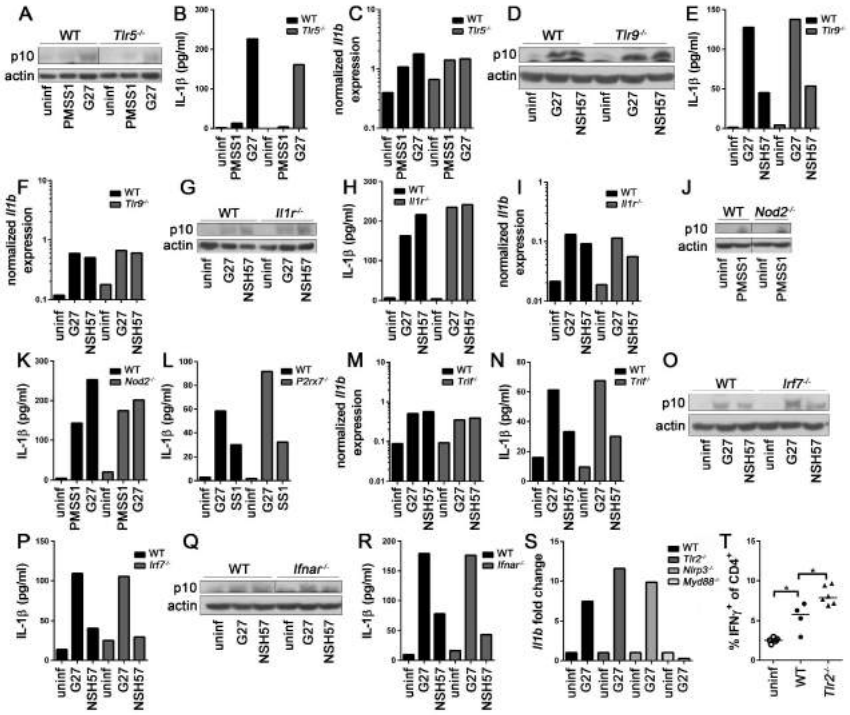
Supplemental Figures

Supplemental Figure 1



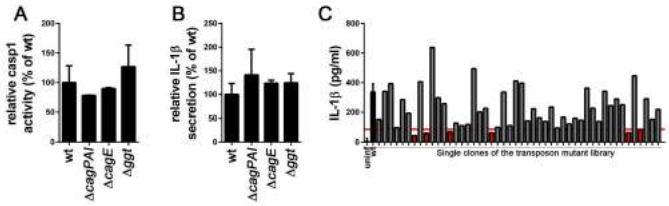
Suppl. Figure 1: IL-1β secretion by *H. pylori*-infected DCs depends on NLRP3 and ASC, but is independent of NLRC4, NLRP6 and AIM2. (**A-E)** BMDCs generated from mice of the indicated genotypes were infected overnight with *H. pylori* NSH57 and G27 at an MOI of 50, with or without prior *E. coli* LPS stimulation. **(A and B)** IL-1β ELISA of samples generated as shown in main Figure panels 1B and 1D, but without prior *E. coli* LPS stimulation. Pooled data of 3 independent experiments are shown (n=3). **(C)** WB analysis of caspase-1 activation (p10) in the cell supernatant compared to full length caspase-1 (p45) and GAPDH in the cell extract. One representative experiment of 3 is shown (n=3). **(D and E)** IL-1β ELISA of samples stimulated with 5ng/ml *E. coli* LPS for 3h prior to infection **(D)** or w/o prestimulation **(E)**. Pooled data of 3 independent experiments (n=3) are shown. Data represent mean ± SD; statistics: Mann-Whitney *U* test.

Supplemental Figure 2



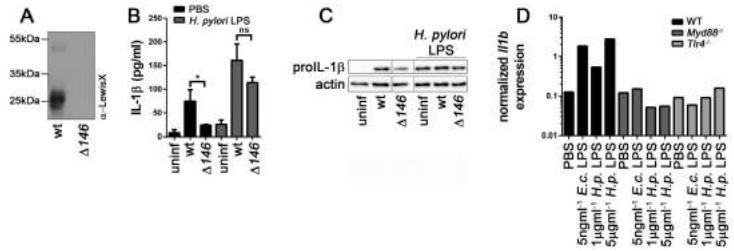
Suppl. Figure 2: Caspase-1 activation and IL-1β secretion by *H. pylori*-infected DCs is independent of TLR5 and TLR9, IL-1R, Nod2, P2X7R and the TRIF-IRF7-IFNaR axis. (A-R) BMDCs generated from mice of the indicated genotypes were infected overnight at an MOI of 50 with *H. pylori* NSH57, G27, SS1 and/or PMSS1. **(A, D, G, J, O and Q)** WB analysis of caspase-1 activation (p10) in the cell supernatant compared to actin expression in the cell extract. Lanes in the p10 WB in panels A, J and Q were run on the same gel, but were noncontiguous. **(B, E, H, K, L, N, P and R)** IL-1β secretion was analyzed by IL-1β ELISA of cell supernatants. **(C, F, I and M)** *Il1b* transcription was measured by qRT-PCR (normalized to *Gapdh*). **(S)** *Il1b* transcription in spleen DCs was measured by qRT-PCR and normalized to *Gapdh* and to uninfected controls. **(T)** MLN single cell suspensions derived from individual mice were re-stimulated with PMA/ionomycin and stained for IFNγ and CD4. Representative experiments are shown throughout (A-I, L, O, P and T: n=2); J and K: n=4; M, N, and Q-S: n=3). Each symbol represents one mouse. Horizontal lines indicate the median; statistics: Mann-Whitney *U* test.

Supplemental Figure 3



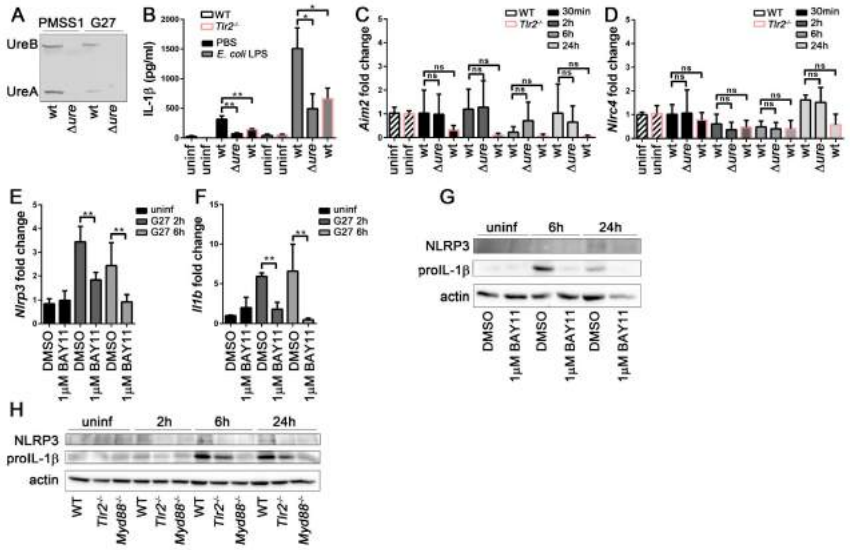
Suppl. Figure 3: IL-1β secretion by *H. pylori*-infected DCs is independent of known *H. pylori* virulence factors. **(A and B)** BMDCs generated from wild type mice were infected overnight with *H. pylori* G27 wild type and the indicated mutants and assessed with respect to caspase-1 activation (by quantification of Western blot signals for the p10 subunit, **(A)**) and IL-1β secretion (by ELISA, **(B)**). At least two and up to four experiment are pooled in A and B. Data represent mean ± SD. **(C)** Representative 96 well plate IL-1β ELISA result of the transposon mutant library screen, where all clones (in red) exhibiting IL-1β expression levels under the 75% reduction cut-off (indicated by the horizontal line) were selected for a second round of analysis.

Supplemental Figure 4



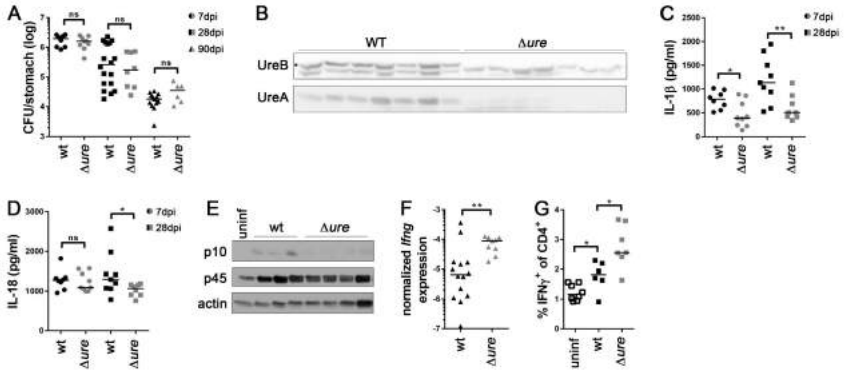
Suppl. Figure 4: *H. pylori* LPS induces the transcription of *Il1b* via TLR4 and MyD88. (A) Western blot of Lewis antigen expression of wild type G27 and the Δ*146* mutant strain. One representative blot of 3 is shown (n=3). **(B)** Wild type BMDCs were infected overnight with *H. pylori* G27 wild type and the Δ*146* mutant in the absence or presence of 1μg/ml purified *H. pylori* LPS, and assessed with respect to IL-1β secretion by ELISA. Pooled data of 3 independent experiments are shown (n=3). Data represent mean ± SD; statistics: Mann-Whitney *U* test. **(C)** BMDCs were treated as described in B and analyzed for proIL-1β expression by Western blotting of cell extract (lanes were run on the same gel, but are not contiguous). One representative experiment of 2 is shown (n=2). **(D)** Wild type, *Myd88*$^{-/-}$ and *Tlr4*$^{-/-}$ BMDCs were treated overnight with either purified *E. coli* or *H. pylori* LPS at the indicated concentrations and assessed with respect to *Il1b* expression by qRT-PCR (samples were normalized to *Gapdh*). One representative experiment of 2 is shown (n=2).

Supplemental Figure 5



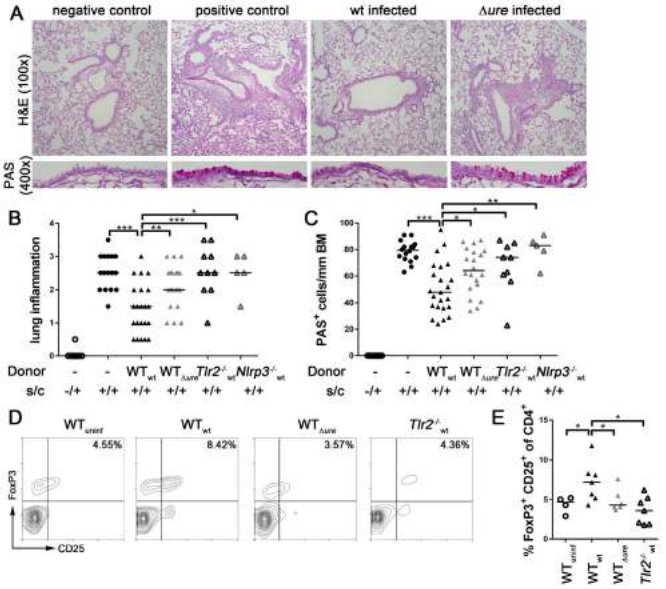
Suppl. Figure 5: Urease-proficient *H. pylori* induces the transcription of *Nlrp3*, but not of *Aim2* and *Nlrc4*, via TLR2, MyD88 and NF-κB. (A) UreA- and UreB-specific Western blot of extracts generated from PMSS1 and G27 wild type and Δ*ure* strains. One representative blot of 3 is shown (n=3). **(B-H)** BMDCs of the indicated genotypes were infected overnight with *H. pylori* G27 wild type and the Δ*ure* mutant as indicated and assessed with respect to IL-1β secretion by ELISA, and proIL-1β, AIM2, NLRC4 and NLRP3 expression by Western blotting and/or qRT-PCR. **(B)** IL-1β ELISA of the 24h-infected samples shown in Figure 2I, along with parallel samples that were additionally pre-stimulated with *E. coli* LPS for 3h. Pooled data of 3 independent experiments are shown (n=3). **(C and D)** qRT-PCR for *Aim2* **(C)** and *Nlrc4* **(D)** of the samples shown in Figure 2I. Pooled data of 4 independent experiments are shown (n=4). **(E-G)** Wild type BMDCs were treated with DMSO or the NF-κB inhibitor BAY11 for 1h prior to infection with wild type G27. Transcription of *Nlrp3* **(E)** and *Il1b* **(F)** was analyzed at the indicated time points using qRT-PCR (normalized to *Gapdh* and to completely untreated, uninfected controls). Pooled data of 3 independent experiments are shown in E and F (n=3). **(G)** NLRP3 and proIL-1β expression as analyzed by Western blotting of cell extracts relative to actin as loading control. One representative experiment of 3 is shown (n=3). **(H)** WT, *Tlr2*$^{-/-}$ and *Myd88*$^{-/-}$ BMDCs were infected for the indicated time points with wild type G27; NLRP3 and proIL-1β expression was analyzed by Western blotting of cell extracts relative to actin as loading control. One representative experiment of 3 is shown (n=3). Data in B-F represent mean ± SD; statistics: Mann-Whitney *U* test.

Supplemental Figure 6



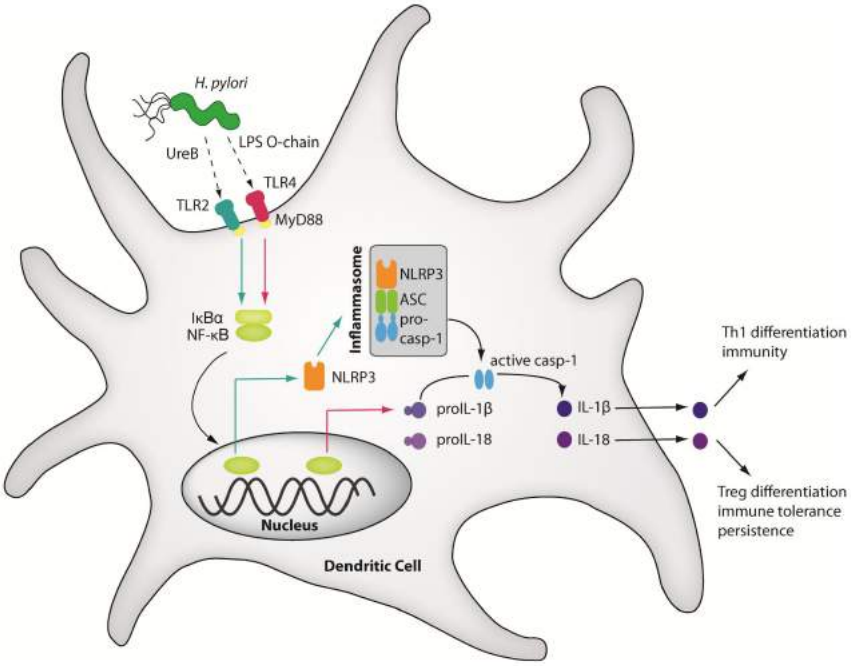
Suppl. Figure 6: *H. pylori* urease is required for inflammasome activation in adult-infected mice. **(A-G)** Mice were infected at 6 weeks of age with *H. pylori* PMSS1 wild type or the Δ*ure* mutant. **(A)** Gastric *H. pylori* colonization at the indicated time points post infection, as determined by plating and colony counting. **(B)** UreA- and UreB-specific Western blot of *H. pylori* re-isolates recovered after 28 days of infection. Each lane represents pooled re-isolates from one animal. **(C and D)** IL-1β **(C)** and IL-18 **(D)** ELISA of gastric mucosal homogenates obtained at the indicated time points post infection. Each symbol represents one animal. **(E)** Western blotting analysis of activated caspase-1 p10, full-length caspase-1 p45 and actin as loading control of gastric mucosal homogenates after 28 days of infection. Each lane represents one animal. **(F)** *Ifng* expression in the gastric mucosa after 90 days of infection, as measured by qRT-PCR and normalized to *Gapdh*. **(G)** MLN single cell suspensions derived from individual mice were re-stimulated with PMA/ionomycin and stained for IFNγ and CD4. Each symbol represents one mouse. Horizontal lines indicate the median; statistics: Mann-Whitney *U* test. Pooled data from 2 independent experiments are shown throughout (n=2); note that for technical reasons not every parameter could be analyzed for each mouse.

Supplemental Figure 7



Suppl. Figure 7: TLR2, NLRP3 and *H. pylori* urease are required for the generation of asthma-suppressing Tregs. (A) Representative images of H&E- (upper panel, magnification 100x) and PAS- (lower panel, magnification 400x) stained sections of the lungs described and scored in main Figure 3H and 3I. **(B and C)** Neonatal wild type, *Tlr2*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were infected with either wild type PMSS1 or its Δ*ure* mutant for 28 days. CD4^{+}CD25^{+} Tregs were immunomagnetically isolated from Peyer's patches and MLNs of these donor mice and adoptively transferred i.v. to ovalbumin-sensitized wild type recipients one day before ovalbumin challenge (s/c, sensitized/challenged). Lung inflammation **(B)** and goblet cell metaplasia **(C)** was quantified on H&E- and PAS-stained tissue sections, respectively (BM, basement membrane). Pooled data of 3 experiments are shown (n=3). **(D and E)** Neonatal wild type and *Tlr2*$^{-/-}$ mice were infected with either wild type PMSS1 or the Δ*ure* mutant for 28 days. MLNs were isolated and stained for CD4, CD25 and FoxP3. Representative FACS plots of the CD4^{+} gate **(D)** are shown along with quantitative data for all animals **(E)**. Horizontal lines indicate the median; statistics: Mann-Whitney *U* test. Data in D and E are representative of 2 independent experiments (n=2).

Supplemental Figure 8



Suppl. Figure 8: Model of *H. pylori*-induced inflammasome activation. *H. pylori* LPS and the urease B subunit (UreB) collaborate to promote NLRP3 inflammasome and caspase-1 activation as well as IL-1β and IL-18 processing and secretion. *H. pylori* LPS signals via TLR4, MyD88 and NF-κB to activate *Il1b* transcription (indicated by red arrows), whereas UreB signals via TLR2, MyD88 and NF-κB to activate *Nlrp3* transcription (green arrows). The assembly of NLRP3, ASC and pro-caspase-1 is triggered through an as yet unknown mechanism leading to caspase-1 activation, and to the processing of proIL-1β and proIL-18. The mature cytokines are released and promote Th1 differentiation and *H. pylori* clearance in the case of IL-1β, and Treg differentiation, immune tolerance and persistence in the case of IL-18. Note that the pro-form of IL-18 is constitutively expressed in DCs. While shown representatively for a dendritic cell, other immune cells and gastric epithelial cells may activate the NLRP3 inflammasome in a similar manner upon exposure to *H. pylori* and may contribute as much or more mature IL-1β and IL-18 to the overall cytokine levels in the infected gastric mucosa.

Supplemental Table 1

gene #	gene name	# of hits
LPS synthesis		
HPG27_38	mannose-6-phosphate isomerase	3
HPG27_39	GDP-D-mannosedehydratase	4
HPG27_104	L-fuculose 1-phosphate aldolase	1
HPG27_146	lipopolysaccharide1,2-glycosyltransferase	5
HPG27_437	DD-heptosyl transferase	1
Urease gene cluster		
HPG27_67	ureaseB	3
HPG27_68	ureaseA	5
Restriction-modification system		
HPG27_436	type II methyltransferase	1
HPG27_746	putative type I R-M enzyme	1
HPG27_806	type I restriction enzyme M protein	1
HPG27_945	phage/colicin/tellurite resistance cluster Y protein	1
HPG27_1316	type III restriction enzyme R protein	1
HPG27_1328	putative type III restriction enzyme R	2
HPG27_1444	type III R-M system modification enzyme	1
Chemotaxis		
HPG27_95	methyl-accepting chemotaxis protein	8
Outer membrane proteins		
HPG27_739	outer membrane protein	1
HPG27_1501	putative outer membrane lipoprotein	1
Hypothetical proteins		
HPG27_773	hypothetical protein	1
HPG27_936	hypothetical protein	1
HPG27_1131	hypothetical protein	1
HPG27_1133	hypothetical protein	1
HPG27_1275	hypothetical protein	1
HPG27_1282	hypothetical protein	1
HPG27_1358	hypothetical protein	1
Others		
HPG27_487	cag pathogenicity island protein X	1
HPG27_603	putative 3-hydroxy acid dehydrogenase	1
HPG27_772	flagellar rotor protein	1
HPG27_1129	alpha carbonic anhydrase	1
HPG27_1138	aldo-keto reductase	1
	plasmid	1
	Different non coding regions	9
	No sequences due to growth deficit	2

Supplemental methods

H. pylori *strains, infection of DCs and transposon library screening*

The following *H. pylori* strains were used for *in vitro* infection of BMDCs. Strains G27, SS1 and PMSS1 have been described previously (1-3). NSH57 is a mouse-adapted derivative of strain G27 that was generated by three 3-week passages in FVB mice (4). To create the transposon library used here (generously donated by Nina R. Salama, Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, USA), genomic DNA prepared from the original 10,000-clone G27 library (5) was transformed into NSH57 by natural transformation (4). For the analysis of individual clones with respect to their induction of IL-1β secretion by BMDCs, bacteria were cultured from frozen stocks on horse blood agar supplemented with 25 μg/ml of chloramphenicol at 37°C for 2 days under microaerophilic conditions. Colonies were picked and expanded individually o/n on fresh horse blood plates prior to inoculation of 100μl liquid cultures (Brucella broth (Difco) containing 10% FBS (Life Technologies)) in 96 well format. Liquid cultures were grown for 6h with shaking at 37°C and used for BMDC infection in 96 well flat bottom plates and for cryopreservation of individual clones. The supernatants of infected BMDCs were subjected to IL-1β ELISA after 16h of co-culture. For sequencing of the transposon flanking regions, the following primer was used: 5'-CAG TTC CCA ACT ATT TTG TCC-3'. The screen was saturated after examination of ~2500 mutants. All *H. pylori* liquid cultures were routinely assessed by light microscopy for contamination, morphology, and motility prior to use in infections.

Generation and infection of BMDC, spleen DCs and human blood-derived DCs

For the generation of BMDCs, single cell suspensions were prepared from hind leg bone marrow and seeded at 10^5 cells per well in 96 well plates for ELISA or at $2x10^6$ cells per well in 6 well plates for Western blotting in RPMI/10% FCS and 4ng/ml GM-CSF and cultured for 6 days. BMDCs were infected for 16h with *H. pylori* at a multiplicity of infection of ~50. BMDCs were pretreated for 3h with 5ng/ml LPS (Sigma Aldrich) to stimulate *Il1b* expression where indicated. BAY11-7082 (Sigma Aldrich) was added at 1μM final concentration 1h prior to infection of BMDCs to inhibit NF-κB signaling. For the isolation of splenic DCs, spleen cell suspensions were prepared by digestion using 1mg/ml CollagenaseD and 0.1% DNaseI (both from Roche) in serum-free RPMI for 1h at 37°C. DCs were immunomagnetically isolated using CD11c MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions and seeded at $2x10^5$ cells per well in 96 well round bottom plates. For the generation of human DCs, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy volunteer blood donors obtained from the blood donation center Zürich (ZHBSD) using Ficoll-Paque™ (GE Healthcare). $CD14^+$ monocytes were isolated from PBMCs using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions and cultured in CellGro DC medium (Cellgenix) supplemented with 1000U/ml GM-CSF and 500IU/ml IL-4 (both R&D Systems) for 6 days.

Induction and quantification of allergic asthma

Ovalbumin model: Mice were sensitized by i.p. injection of 20µg ovalbumin (Sigma-Aldrich) emulsified in 2.25mg aluminum hydroxide (Alum Imject; Pierce) at 6 and 8 weeks of age. Sensitized mice were challenged with 1% aerosolized ovalbumin using an ultrasonic nebulizer (NE-U17; Omron) for 20 min daily on days 31, 32 and 33 post initial sensitization. For Treg transfer experiments, neonatally infected donor mice were sacrificed at the age of 5 weeks and Tregs were immunomagnetically isolated from MLN and Peyer's Patches single cell suspensions ($CD4^+CD25^+$ mouse regulatory T cell isolation kit; R&D). $1x10^6$ Tregs were injected per animal into sensitized wild type recipient mice one day before the first allergen challenge. House dust mite (HDM) model: Mice were sensitized by intranasal administration of 1µg HDM (Greer) at 6 weeks of age. Mice were challenged with 10µg HDM per animal on five consecutive days, starting on day 8 after sensitization. Anti-IL-18 antibody (YIGIF74-1G7, BioXCell) was applied weekly (50µg per dose) starting at 7 days of age. After sacrifice, lungs were lavaged via the trachea with 1ml PBS. Bronchoalveolar lavage fluid (BALF) cells were counted using trypan blue dye exclusion. Differential eosinophil counts were performed on cytocentrifuged preparations stained with the Microscopy Hemacolor Set (Merck). Lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin. Tissue sections were stained with H&E and periodic acid-Schiff and examined in blinded fashion on a BX40 Olympus microscope. Peribronchial inflammation was scored on a scale from 0 to 4. PAS-positive goblet cells were quantified per 1mm of basement membrane. For lung re-stimulation, single cell suspensions were prepared by collagenase 1A (Sigma Aldrich) digestion for 45min at 37°C. Cells were seeded at $5x10^5$ cells per well and stimulated with 250µg/ml ovalbumin for 3 days. Supernatants were analyzed for secretion of IL-5 by ELISA (eBioscience) according to the manufacturer's instructions. For detection of HDM-specific IgE, plates were coated with 25µg/ml HDM antigen; HDM-bound IgE was detected using α-mouse IgE-HRP (GTX77227, GeneTex).

Western Blotting and ELISA of cell culture supernatants and extracts, and of gastric mucosal homogenates

To detect proteins in cell culture supernatants, 500µl of supernatants were TCA-precipitated and subjected to Western blotting. Cell extracts and extracts of scraped and shock frozen murine gastric mucosa were prepared using RIPA-Buffer supplemented with protease inhibitors (complete Mini, Roche). For lysis of bacteria, 200µg/ml lysozyme (Sigma Aldrich) was added to the lysis buffer. For Lewis antigen detection, bacterial lysates were treated with 125ng/ml Proteinase K (Macherey-Nagel) o/n. The following antibodies were used: α-Casp1 p10/p45 (sc514, Santa Cruz), α-NLRP3 (ab91525, abcam), α-IL-1β (AF-401-NA, R&D Systems), α-actin (sc1616, Santa Cruz), α-GAPDH (G9545, Sigma Aldrich), α-*H. pylori* Urease (ab51954, abcam), α-LewisX (ab3358, abcam). Cytokines in gastric mucosal extracts and the supernatants of infected BMDCs or splenic DCs were analyzed by ELISA (human IL-

1β and mouse IL-1β, both BD Biosciences; IL-18, eBioscience) according to the manufacturers' instructions.

Real time qRT-PCR of cytokines in cell extracts and gastric mucosal homogenates

For real-time RT-PCR of shock frozen stomach mucosa or BMDC cell pellets, total RNA was isolated using NucleoSpin RNA II kits (Macherey-Nagel). For isolation of RNA from splenic DCs, the RNeasy microkit (Qiagen) was used. RNA was reversely transcribed into cDNA using superscript III (Invitrogen). The corresponding cDNA served as a template for real-time PCR performed using the LightCycler 480 SYBR Green I master kit (Roche). Samples were normalized to GAPDH expression (conditions: Tm 60°C, 50 cycles; primers: GAPDH fw: 5'-GAC ATT GTT GCC ATC AAC GAC C-3' / GAPDH rv: 5'-CCC GTT GAT GAC CAG CTT CC-3', pro-IL-1β fw: 5'-TTG ACG GAC CCC AAA AGA TG-3' / pro-IL-1β rv: 5'-TGG ACA GCC CAG GTC AAA-3', NLRP3 fw: 5'-CCC TTG GAG ACA CAG GAC TC-3' / NLRP3 rv: 5'-GGT GAG GCT GCA GTT GTC TA-3', IFNγ fw: 5'-ATC TGG AGG AAC TGG CAA AA-3' / IFNγ rv: 5'-TTC AAG ACT TCA AAG AGT CTG AGG TA-3', AIM2 fw: 5'-CAG GCA ATT GCA TCT GAG AG-3' / AIM2 rv: 5'-CGC CTC ACA AAG ATT TTC ACT-3', NLRC4 fw: 5'-GAA GAA TCC TGT GAT CTC CAA GAG-3' / NLRC4 rv: 5'-GAT CAA ATT GTG AAG ATT CTG TGC-3').

Single cell preparations of MLN and Peyer's patches and FACS staining

Single cell suspensions of MLNs and Peyer's patches were prepared by collagenaseIV (Sigma Aldrich) digestion for 25min at 37°C. For detection of intracellular cytokines, cells were seeded at $2x10^5$ cells per well and treated with 100nM PMA (Sigma Aldrich), 1μg/ml Ionomycin (Sigma Aldrich) and 2μg/ml Monensin (Enzo LifeScience) for 4h. Fixation and permeabilization was performed with the Cytofix/Cytoperm™ Kit (BD Bioscience). The following antibodies were used for staining: CD4-FITC (RM4-5, Biolegend), CD25-Biotin (MAGM208, PartNo 860126, R&D), Streptavidin-eFluor450 (48-4317-82, eBioscience), IFNγ-PECy7 (XMG1.2, BD Biosciences), IL-17-APC (TC11-18H10.1, Biolegend) and FoxP3-APC (FJK-16s, eBioscience).

Cloning, expression and purification of recombinant proteins

UreaseA and UreaseB gene were amplified from bacterial genomic DNA using pfu-DNA polymerase (ThermoScientific) with the following primers: UreA fw: 5'-ATA TGA ATT CTT AAT TCT CCT TAA TTG TTT TT-3', UreA rv: 5'- ATA TGG ATC CAA ACT CAC CCC AAA AGA-3', UreB fw: 5'- ATA TGT CGA CCG AAC ACA TGG TAA GTT T-3', UreB rv: 5'- ATA TGA ATT CAA AAA GAT TAG CAG AAA AGA-3'. Amplified fragments were inserted into the pGEX-4T-1 plasmid (GE Healthcare) using BamHI and EcoRI restriction sites for UreaseA and SalI and EcoRI restriction sites for UreaseB (all restriction enzymes obtained from New England Biolabs). After amplification and sequencing

of the resulting plasmids, positive clones were transformed into *E. coli* BL21. Induction of overexpression of the GST-tagged proteins was done by the addition of 1mM IPTG. Bacterial cells were homogenized by sonication and precleared lysates were applied to GST GraviTrap columns (GE Healthcare). Tagged proteins were isolated according to manufacturer's instructions.

Supplemental references

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